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13. ABSTRACT ***(Maximum 200 Words)***
The study of the molecular mechanisms that control cell growth and motility in prostate cancer is an under-investigated area of research, whereas significant progress has recently been made in breast cancer in this field. Members of the Integrin family of cell adhesion receptors, have emerged as crucial regulators of proliferation, migration, intracellular signaling and gene induction. The objective of this study was to investigate the effect of target molecules: β_1 and β_3 integrins and their downstream signaling pathways on prostate cancer growth and motility. The proposal has highlighted a new approach to the understanding and control of prostate cancer growth and metastatic lesions. Indeed, the PI has been the first to describe a role for β_{1C} integrins in prostate cancer cell proliferation and for β_3 integrins in prostate cancer cell migration. This study has, thus, allowed new target validation in prostate cancer therapy, thus, meeting the goals of the USAMRMC Prostate Cancer Research Program (PCRP).

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Final Report for Award Number DAMD 17-98-1-8506
Title: β1 and β3 Integrins in Prostate Cancer
Period: July 1998- December 2002
PI: Lucia R. Languino, Ph.D.

NOTE: Please note that the PI has moved from Yale University to the University of Massachusetts, Worcester, MA in August 2002. As a consequence, some time has been lost and a one-year no-cost extension has been granted by the DOD to the PI to recover the loss of time due to the transfer. The transfer to the U. Massachusetts has not changed the Statement of Work of this proposal.

PERSONNEL

Lucia R. Languino, Mara Fornaro, Duo-Qi Zheng, Giovanni Tallini, Dhanpat Jain, Thomas Manes, Loredana Moro at Yale University.

Lucia R. Languino, Michael King at University of Massachusetts.

INTRODUCTION

Abnormal prostate cell proliferation and motility are responsible for the development of prostate cancer. Although very little is known about the basic molecular mechanisms that are responsible for abnormal prostate growth and contribute to this disease, it has been shown that prostate cell growth depends on cell-extracellular matrix interactions (Fornaro et al., 2001). Adhesive contacts between cells and extracellular matrix components are mediated by *integrins*, the most widely distributed gene superfamily of adhesion receptors. Strong evidence regarding the role of integrins and their ligands in cancer cell growth and motility, derive from studies performed *in vitro* and *in vivo*.

β1C and β1A Integrins The β_{1C} (formerly β_{1S}) integrin, a variant cytoplasmic domain form of the β_1 integrin, has been discovered and characterized by the applicant(Fornaro et al., 1995) (Fornaro et al., 1996) (Fornaro et al., 1999). Recent studies conducted by the applicant group and by others have discovered β_{1C} powerful inhibitory effect on cell proliferation; in contrast, the variant β_{1A} promoted cell proliferation. This proposal is based on the hypothesis that the regulated expression of different integrin variant cytoplasmic domains might be a mean to modulate cell proliferation *in vitro* and *in vivo*.

β3 Integrin A positive role of the β_3 integrin in cell migration as well as in tumor (specifically melanoma) growth has been established *in vitro* and *in vivo* (Zheng et al., 1999). Furthermore, $\alpha_v\beta_3$ expression correlates with a more metastatic phenotype in melanoma cells and bone-residing breast metastases express high levels of β_3, indicating a role for this integrin in *in vivo* metastatic lesion establishment and growth.

Although these studies strikingly define β_{1C}, β_{1A} and β_3 integrins as growth and motility modulators, studies on their distribution and functions in prostate cancer cells were not available, when the present proposal was submitted, except for the applicant report by Fornaro et al, 1996. The PI, thus, hypothesized in the present proposal that the down-regulation or loss of expression of β_{1C} and the upregulation of β_3, observed in prostate cancer cells, play a crucial role in the

modulation of prostate cell proliferation and motility *in vitro* and *in vivo* through unique intracellular signaling pathways. Objective of this study was to investigate the effect of target molecules: β1 and β3 integrins and their downstream signaling pathways on prostate cancer growth and motility. It is specifically planned to study the effect 1) of β1C on prostate cancer cell growth in vitro and tumor growth in vivo and 2) of β3 on prostate metastasis establishment and growth in SCID mice.

BODY

As indicated in the approved Statement of Work, the following aspects of the project have been analyzed between July 1998 and December 2002 of this proposal and the following accomplishments have been made.

Task 1.To evaluate the role of β1C and β1A integrins in the modulation of Prostate Cancer Primary Cell proliferation *in vitro* by using transfectants expressing the individual subunits or their mutant forms.

a) Evaluate the expression of β1C, β1A and β3 integrins in Prostate Cancer and normal tissues by RNA and Protein analysis

The PI has analyzed the expression of the two variant forms of the β1 subunits, β1C and β1A, that respectively inhibit or stimulate cell proliferation (Fornaro et al., 1995), in prostate cancer tissue specimens. First, immunohistochemical analysis performed using affinity-purified antibodies specific for β1C, had demonstrated that β1C is expressed in normal or benign tissue and is downregulated in comparison to the benign tissue in 34 tumor specimens exhibiting different Gleason's patterns (Fornaro et al., 1996) (Fornaro et al., 1998) (Fornaro et al., 1999). A very high correlation of β1C and $p27^{kip1}$ expression was found in 93% of benign cells and in 84-91% of neoplastic cells of the analyzed specimens (p<0.0001). In 75% of the specimens analyzed, both β1C and $p27^{kip1}$ were downregulated in tumor areas in comparison to benign counterparts. In contrast to β1A, forced expression of β1C *in vitro* was accompanied by an increase in $p27^{kip1}$ levels, by inhibition of cyclin A-dependent kinase activity and of the Ras/MAP kinase pathway. Furthermore, β1C sensitized cells to drug-induced apoptosis. β1C inhibitory effect on cell proliferation and survival was completely prevented by $p27^{kip1}$ antisense or by expression of activated Ras and MAP kinase (Fornaro et al., 1999) (Fornaro et al., Abstract, 2001).

Then, we have studied β1C and β1 integrin expression at both mRNA and protein levels by northern and immunoblotting analysis using freshly isolated neoplastic and normal human prostate tissue specimens (Perlino et al., 2000). Steady-state mRNA levels were evaluated in thirty-eight specimens: thirty-three prostatic adenocarcinoma exhibiting different Gleason's grade and five normal tissue specimens that did not show any histological manifestation of benign prostatic hypertrophy. Our results demonstrate that β1C mRNA is expressed in normal prostate

and is significantly downregulated in neoplastic prostate specimens. In addition, using a probe that hybridizes with all β_1 variants, mRNA levels of β_1 are found reduced in neoplastic versus normal prostate tissues. We demonstrate that β_{1C} mRNA downregulation does not correlate with either tumor grade or differentiation according to Gleason's grade and TNM system evaluation, and that β_{1C} mRNA levels are not affected by hormonal therapy. In parallel, β_{1C} protein levels were analyzed. As expected, β_{1C} is found to be expressed in normal prostate and dramatically reduced in neoplastic prostate tissues; in contrast, using an antibody to β_1 that recognizes all β_1 variants, the levels of β_1 are comparable in normal and neoplastic prostate, thus indicating a selective downregulation of the β_{1C} protein in prostate carcinoma. These results demonstrate for the first time that β_{1C} and β_1 mRNA expression is downregulated in prostate carcinoma, whereas only β_{1C} protein levels are reduced (Perlino et al., 2000). Our data highlight a selective pressure to reduce the expression levels of β_{1C}, a very efficient inhibitor of cell proliferation, in prostate malignant transformation.

In parallel, the expression of the β_3 integrin was analyzed in 21 metastatic (lymph nodes and bone) prostate cancer lesions. In a manuscript in preparation, using tissue specimens from prostatectomies in a semi-quantitative analysis, expression of $\alpha v\beta 3$ integrin was evidentiated only in cancer cells but it was absent in normal glands. $\alpha v\beta 3$ was not expressed in any of the examined 5 autopsy tissue specimens from individuals (age 20-40 years old) that did not show functional or morphological prostate alterations. The immunohistochemical analysis was performed using three different mAbs (AP-3, SSA6, SZ21), previously described (Gladson and Cheresh, 1991; Gladson et al., 1996) to recognize $\alpha v\beta 3$ in paraffin-embedded and formalin-fixed tissue sections with consistent results. To investigate whether $\alpha v\beta 3$ was expressed in metastatic lesions, 21 (bone and lymph node) metastatic specimens were stained for $\alpha v\beta 3$. The results indicate that 15 out of 21 prostate cancer metastases showed significant expression of $\alpha v\beta 3$.

b) Transient transfection in primary culture of human prostate epithelial cells using chicken specific epitope in human β_1 integrin subunit constructs including the use of an adenovirus-based expression system to express human β_1 integrin variants.

Chimeric constructs have been generated (see Task 2a) , however, their expression in primary cultures has been difficult to detect. Therefore, it was decided to analyze the effect of β_1 integrin variants in human prostate cancer cells. Specifically, as described below, PC3 and LNCaP cells have been used to generate stable transfectants expressing β_{1A} or β_{1C} or β_3.

c) Test the ability of β_1 to inhibit prostate cancer cell proliferation using ^{3}H-thymidine incorporation.

The ability of β_{1C} and β_{1A} to inhibit prostate cancer cell proliferation has been confirmed using ^{3}H-thymidine incorporation and a new assay that utilizes SRB staining.

The ability of chicken/human β_{1C} to inhibit PC3 cell growth, was assessed in *in vitro* proliferation assays. PC3 stable cell lines were plated (35,000 cells/well) on 24-well plates. Cells were cultured for 96 h in growth medium either in the absence or in the presence of 1

μg/ml tetracycline, and either in the absence or in the presence of 10% fetal calf serum (FCS). Cells were then detached using 0.05% trypsin/0.53 mM EDTA. Cells were washed, resuspended in growth medium and counted and viability determined using trypam blue exclusion. Triplicate observations were performed. The results show that induction of β1C expression in PC3 stable cell transfectants resulted in strong inhibition of cell proliferation in response to serum as compared to either β1A- or mock- transfected cells (Table 1).

Table 1. β1C Effect on Cell Proliferation

Cell Line	% Inhibition[a]
PC3-β1C	55 ± 4.7*
PC3-β1A	2.36±6.6*
PC3-mock	0*

[a] Data are expressed as mean±SEM % inhibition in cell proliferation in the presence of 10% FCS in two to four experiments. The percentage of growth inhibition was calculated as follows: (total number of cells cultured in the absence of tetracycline/total number of cells cultured in the presence of tetracycline) x 100. Group differences were compared using t-test. * The differences in the inhibition of cell proliferation between PC3-β1C and PC3-β1A, between PC3-β1C and PC3-mock stable cell transfectants in the presence of 10% FCS are statistically significant ($p<0.001$)

d) Identify crucial residues in the β1C cytoplasmic domain responsible for inhibiting cell growth using genetically engineered transfectants carrying single point mutations and test β1C inhibitory effect on cell proliferation.

The generation of β1C cytoplasmic domain mutants is in progress.

Task 2. To evaluate the role of β1C and β1A integrins in the modulation of Prostate Cancer growth in *in vivo* experimental systems (nude mice) by using stable transfectants expressing the individual subunits.

a) Obtain inducible β1 expression in prostate cell lines.

The PI has generated PC3 stable cell lines expressing either β1C or β1A under the control of a tetracycline regulated promoter and shown that β1C, but not β1A, blocks cell proliferation in vitro (Table 1) and tumor growth in vivo (Table 2; manuscript in preparation; a reprint will be submitted when published). These results have been obtained using the following procedures. In order to transfect human prostate epithelial cells with human β1 integrin isoforms, the PI has generated chimeric cDNAs consisting of the chicken β1 extracellular and transmembrane domain and either human β1C or human β1A cytodomain sequences by PCR-driven splice overlap extension. These chimeric constructs allow to discriminate between endogenous β1 integrin and exogenous β1C and β1A integrin variants in human cells.

PCR reactions were performed using an automatic thermal cycler (Perkin-Elmer Cetus). The conditions used for the PCR reactions were: denaturation at 95°C for 1 minute, annealing at 45°C for 1 minute and extension at 72°C for 1 minute. Thirty cycles of amplification were used. The amplification was performed in 1x *Vent* polymerase buffer (New England Biolabs), 200 μM each dNTP (Perkin-Elmer Cetus), 0.15 μM each primer, 2 mM $MgSO_4$ and 0.5 U of *Vent* polymerase (New England Biolabs). Primers (see attached Table 1) were designed to amplify chicken β1 subunit from nucleotides 1787 to 2365 using chicken β1 cDNA template. Additional primers were designed to amplify either human β1A cytodomain sequences from nucleotides 2357 to 2497 or human β1C cytodomain sequences from nucleotides 2357 to 2613 (Languino and Ruoslahti, 1992) using either human β1A or human β1C cDNA template, respectively. The chicken reverse primer and the human forward primer corresponded to a region that was identical between the species and were complementary to each other. The amplified chicken and human cDNA fragments were mixed together and subjected to an additional amplification using the chicken forward and the human reverse primers. The resulting chimeric cDNA fragments were subcloned into pCR 2.1 vector (Invitrogen) following manufacturer's instructions. The 718-base pair (bp) chicken/human β1A and the 834-bp chicken/human β1C chimeras were excised from pCR2.1 using ClaI and SpeI and subcloned into pTET-Splice (PNAS 92:6522-6526, 1995). The resulting constructs were digested with SalI and ClaI to allow ligation of a DNA fragment corresponding to nucleotides 1 to 1787 of the chicken β1 extracellular domain. The above chicken β1 fragment was isolated from pECE-β1 (provided by Dr. Hynes) using SalI and ClaI restriction enzymes. The chimeric constructs were sequenced by the dideoxynucleotide method to confirm the nature of the chimeric inserts. PC3 cells were electroporated using a Genepulser apparatus (BioRad, Hercules, CA) set at 250 V and 900 μF using either 100 μg pTet-chicken/human β1C or pTet-chicken/human β1A or pTet along with 10 μg pTet-tTA. Neomycin-resistant cells were selected using medium containing 0.2 mg/ml G418 (Life Technologies). G418-resistant clones were isolated and screened for cell surface expression of either chicken/human β1C or chicken/human β1A integrin by FACS using W1B10, monoclonal antibody against the extracellular domain of chicken β1 integrin, or 12CA5, as a negative control, as described (manuscript in preparation; reprints to follow). The results show that comparable levels of surface expression of chicken/human β1C and chicken/human β1A were obtained 72 h after tetracycline removal.

b) Evaluate the role of either β1C or β1A in the modulation of prostate cancer cell growth *in vivo*.

Using genetically engineered and primary prostate cancer cells, we have found that, *in vitro*, the β1 integrins modulate cell adhesion and motility via activation of specific signaling events and via regulation of gene expression. Studies in our laboratory aimed to identify downstream effectors of β1 integrins have unraveled two novel pathways regulated by β1 integrins that involve changes in gene expression: an IGF (insulin-like growth factor)II - mediated pathway that controls cell adhesion (Moro et al., Abstract, 2001) (manuscript in preparation; a reprint will be submitted when published). Therefore, given their deregulated expression in neoplastic cells and their ability to control multiple downstream signals, the β1 integrins and their downstream effectors provide exciting opportunities for new approaches to

cancer therapy.

Because β1C expression significantly inhibits PC3 prostate cancer cell proliferation *in vitro*, the PI has evaluated, in a pilot study, whether expression of β1C reduces tumorigenicity of PC3 *in vivo*. PC3-β1C stable cell transfectants (two clones) were cultured for 72 hours in growth medium either in the absence or in the presence of 1 μg/ml tetracycline. Cells were then detached using 0.05% trypsin/0.53 mM EDTA, washed, and resuspended in RPMI. Cells ($1x10^6$) were inoculated subcutaneously (s.c.) into athymic Balb/c mice (5 mice/group). Mice were given water supplemented with either 5% sucrose or 5% sucrose and 100 μg/ml tetracycline to either induce or prevent β1C expression, respectively. Tumor size was determined using a caliper at day 16 or 14 post-inoculation for clone 1 and 2, respectively. The results show that injection of PC3 cells expressing β1C significantly reduced tumor incidence and growth compared to PC3 cells where expression of β1C was prevented by addition of tetracycline (Table 2; manuscript in preparation; a reprint will be submitted when published).).

Table 2. β1C Effect on Tumor Growth

Cell Line	Tetracycline	Tumor Volume[a]	Tumor Incidence[b]
PC3-β1C clone 1	-	52.5 ± 42.8*	3/5
PC3-β1C clone 1	+	127.2 ± 34.5	4/4
PC3-β1C clone 2	-	108.1 ± 133**	3/5
PC3-β1C clone 2	+	485 ± 364	4/5

[a] Average tumor volume expressed in mm^3±SEM. [b] number of mice bearing tumor.

*The difference in tumor volume between animals injected with PC3-β1C clone 1 cultured in the absence of tetracycline and PC3-β1C clone 1 cultured in the presence of tetracycline are statistically significant (p=0.0206).

** The difference in tumor volume between animals injected with PC3-β1C clone 2 cultured in the absence of tetracycline and PC3-β1C clone cultured in the presence of tetracycline are statistically significant (p=0.0417).

c) Whether β1C expression significantly reduces tumorigenicity of PC3 cells, intratumoral injection of recombinant adenovirus expressing either vehicle or Ad5-β1C or Ad5-β1A will be performed.

This part of the study has not been started yet.

<u>Task 3.To evaluate the role of β3 integrins in the modulation of Prostate Cancer growth and metastatic lesions in in vivo experimental systems (experimental metastasis assays).</u>

a) To generate cells transfected with β3 integrin and β6 integrin

At this time, β_3-LNCaP, and β_6-LNCaP cell transfectants have been generated. The $\alpha_V\beta_3$ integrin has been shown to promote cell migration through activation of intracellular signaling pathways. We describe here a novel pathway that modulates cell migration and that is activated by $\alpha_V\beta_3$ and, as downstream effector, by cdc2 (cyclin-dependent kinase 1, cdk1) (manuscript submitted; a reprint will be submitted when published). We report that $\alpha_V\beta_3$ expression in LNCaP (β_3-LNCaP) prostate cancer cells causes increased cdc2 mRNA levels as evaluated by gene expression analysis, and increased cdc2 protein and kinase activity levels. We provide three lines of evidence that increased levels of cdc2 contribute to a motile phenotype on integrin ligands in different cell types. First, increased levels of cdc2 correlate with more motile phenotypes of several cancer cells. Second, ectopic expression of cdc2 increases LNCaP cell migration, whereas expression of dominant negative cdc2 inhibits migration. Third, cdc2 inhibitors reduce cell migration without affecting cell adhesion. We also show that cdc2 increases cell migration via specific association with cyclin B2 and we unravel a novel pathway of cell motility that involves, downstream of cdc2, caldesmon. Cdc2, cyclin B2 and caldesmon are shown here to localize in membrane ruffles in motile cells. These results show that cdc2 is a downstream effector of the $\alpha_V\beta_3$ integrin, and that it promotes cell migration. This observation will be taken into consideration when analyzing the different phenotypes of these transfectants *in vivo*.

$\alpha_V\beta_3$ expression is detected only in prostate cancer, but not in normal prostate epithelial cells (Zheng et al., 1999). Our recent data suggest that $\alpha_V\beta_3$ integrin and its downstream effector, cdc2, may be important mediators of prostate cancer progression towards an aggressive metastatic phenotype (manes et al., 2002). This claim is supported by our data showing that more metastatic prostate cancer cell variants express higher levels of cdc2 and by data reported by Kallakury et al., indicating that cdc2 is expressed in a majority of prostatic adenocarcinomas and correlates with high Gleason's grade, advanced pathologic stage and metastatic adenocarcinomas (Kallakury et al., 1997). While G1/S cell cycle proteins have been the focus of investigations assessing cell cycle biomarkers in prostate cancer (Kibel and Isaacs, 2000), the results presented here support previous published evidence on the use of cdc2 as a marker with prognostic value. Thus, the functional role of cdc2 in prostate cancer *in vivo* may be different than once thought; it may reflect the migratory, rather than the proliferative, ability of these cells. In conclusion, given their deregulated expression in neoplastic cells and their ability to control multiple downstream signals, the β_3 integrins and their downstream effectors provide exciting opportunities for new approaches to cancer therapy.

We have also described a role for β_3 in activating PI 3-kinase /AKT pathway in prostate cancer cell migration (Zheng et al., 2000). Since constitutive activation of the PI 3- kinase/AKT pathway occurs in cancer cells that become refractory to cytotoxic therapy and higher levels of AKT activation are observed in human prostate cancer cell lines and xenografts versus normal prostate tissues, β_3 integrins and the intracellular signaling pathways stimulated by their engagement may be valid targets for novel drug discovery in prostate cancer therapy.

Construction of integrin chimeras. Two chimeric integrins were expressed in prostate cancer cells: one referred to as β_3/β_6, contains the β_3 extracellular and transmembrane domains

(residues 1 – 741, Zimrin et al, 1988) and a β6 cytoplasmic tail (corresponding to residues 731 to 788 of β6); another, called β6/β3, consists of β6 extracellular and transmembrane domains (residues 1 – 730, Sheppard et al, 1990) and a β3 cytoplasmic tail (corresponding to residues 742 to 788 of β3). β3/β6 integrin chimeras. A recombinant cDNA encoding the β3/β6 chimera was constructed by directional ligation of cDNA and PCR-generated fragments. Identical sequences of β3 and β6 at the transmembrane domain - cytoplasmic domain interface allowed for the generation of cohesive termini between β3 and β6 fragments at this juncture. This was effected by incorporating a restriction enzyme site (Bsa I) that cuts at a distance away from its recognition sequence (GGTCTCN'NNNN') into oligos used in PCR; the cohesive termini are generated upon digestion with Bsa I. Two PCR products, one containing the transmembrane domain from β3 integrin (fragment 1) and one containing the cytoplasmic domain from β6 (fragment 2), were generated by using the following oligos and templates: fragment 1. (β3 forward, nt 2001-2022) GTGACGAGATTGAGTCAGTGAA and (β3 reverse, nt 2218-2239, containing the Bsa-restriction enzyme site) GGTCTCCCAGATGAGCAGGGCGGCAAGG using β3 cDNA in pRc/CMV as template; fragment 2. (β6 forward nt 2410-2431, containing the Bsa-restriction enzyme site) GGTCTCATCTGGAAGCTACTGGTGTCA and (SP6 in vector) ATTTAGGTGACACTATAG using β6 in pcDNA-3 as template. The following gel-purified (QIAEX II) fragments (cDNAs or fragments) were assembled in a ligation reaction with pcDNA-3 digested with EcoRI and XbaI to create a recombinant cDNA encoding the β3/β6 chimera: β3 cDNA digested with EcoRI and AflII + fragment 1 digested with AflII and BsaI + fragment 2 digested with BsaI and XbaI. β6/β3 integrin chimeras. Similarly, a recombinant cDNA encoding the β6/β3 chimera was constructed by directional ligation of cDNA and PCR-generated fragments. The two PCR products, one containing the transmembrane domain from β6 integrin (fragment 3) and one containing the cytoplasmic domain from β3 (fragment 4), were generated by using the following oligos and templates: fragment 3. (β6 forward nt 2060-2081) CCAACCTGTGAACGATGTCCTA and (β6 reverse nt 2395-2416, containing the Bsa-restriction enzyme site) GGTCTCCCAGATGCACAGTAGGACAACC using β6 in pcDNA-3 as template; fragment 4. (β3 forward nt 2231-2253, containing the Bsa-restriction enzyme site) GGTCTCATCTGGAAACTCCTCATCAC and (SP6 in vector) ATTTAGGTGACACTATAG using β3 cDNA in pRc/CMV as template. The following gel-purified (QIAEX II) fragments were assembled in a ligation reaction with pcDNA-3 digested with EcoRI and XbaI to generate a recombinant cDNA encoding the β6/β3 chimera: β6 cDNA digested with EcoRI and BstEII + fragment 3 digested with BstEII and BsaI + fragment 4 digested with BsaI and XbaI. The absence of mutations in all PCR-amplified fragments was verified by sequencing.

CWR22Rv1. Recently, a new human prostate cancer cell line, CWR22Rv1, was derived from a xenograft of a primary tumor serially propagated in mice (Sramkoski et al, 1999). Importantly, this cell line has been shown to form micrometastases in mouse organs normally observed in the human disease, namely, lung, liver, and bone (Holleran et al, 2000); only in the liver, however, were overt metastases observed. FACS analysis has determined that β3 integrin is not expressed by CWR22R (Zheng, unpublished observation). This cell line therefore represents a model system in which to examine the effects of β3 expression in the events associated with the transition of micrometastases to macrometastases. For example, by ectopically expressing β3 in CWR22R, changes in migration, apoptosis and proliferation attributable to β3 expression in micrometastatic cells can be measured. We have recently obtained CWR22Rv1 cells in the

laboratory; we have received the cells by Dr. Culp, have grown them successfully and have shown that they do not express β3 and β6 integrins. The study is therefore, feasible. The use of CWR22Rv1 cells instead of LNCaP cells will not change the goals and objectives of the DAMD 17-98-1-8506 award.

b) Test transfectant cells in vivo in scid mice

To test the possibility that αVβ3 mediates metastatic growth, the use of two mouse models has been planned in this Proposal in collaboration with Drs. Tenniswood (22) and Cher (23). The PI, shows that upon s.c. injection in Matrigel, β3 expression causes minimal tumor growth and micrometastases; whereas upon injection in human bone fragments β3 expression causes significant growth in metastatic lesions (manuscript in preparation; Reprints to follow when published). LNCaP cells in the early phases of metastatic spread are detected in mouse lung (not shown) whereas macrometastases were not evidentiated in any examined organ. It remains to be examined whether αVβ3 promotes progression and growth into macrometastases.

Task 4.To perform a retrospective comprehensive study to evaluate the expression of β1C, β1A integrins and p27kip1 in prostate cancer tissue specimens

a) Immunohistochemical analysis

Prostate tumor tissue microarrays were immunostained with monoclonal and polycloonal antibodies against β1C, β1A and p27. The tumor microarrays consist of clinical prostatic adenocarcinoma specimens and were kindly provided by Drs Bubendorf and Sauter at the department of Pathology of the University of Basel (Switzerland) (Bubendorf L et al J Natl Cancer Inst 1999 91:1758-1764; Bubendorf L et al Cancer Res 1999 59:803-806). Review of the immunostained tissue array sections demonstrates varying degrees of tissue reactivity for β1C. Tissue immunoreactivity is being scored for correlation with clinical and pathologic data (manuscript in preparation; a reprint will be submitted when published).

KEY RESEARCH ACCOMPLISHMENTS

The PI and her group have made significant innovative contributions to the understanding of integrin-mediated mechanisms that control prostate cancer cell growth and migration (see papers and manuscripts by the PI in the Appendix).

The following findings represent the major Research Accomplishments:

- The **β1C** integrin is expressed in benign and is downregulated in neoplastic prostatic epithelium at the protein and mRNA levels. These results indicate that β1C may be a sensitive prognostic indicator of potential high clinical value to predict therapy and patient survival for prostatic adenocarcinoma and that the **β1C** integrin.
- Exogenous expression of **β1C**, but not of **β1A**, completely inhibits thymidine incorporation in response to serum by normal and cancer prostate epithelial cells as well as tumor growth *in vivo*.
- **β3** integrin is expressed in prostatic adenocarcinoma (primary and metastatic lesions).
- **β3** expression increases prostate epithelial cell migration. This study points to $\alpha_V\beta3$ as potential target in prostate cancer cell invasion and metastasis.
- **β1C** functional cytoplasmic domain increases the levels of its downstream effector p27kip1 and inhibits the Ras/MAPK pathway
- FRNK, a negative regulator of FAK functions, inhibits **β3**-LNCaP cell migration on VN.

Also:

- Monoclonal antibodies to **β1C** have been generated. The monoclonal antibodies described here will be useful tools for the study of **β1C**-specific interactions with intracellular proteins and **β1C**-downstream signaling pathways, as well as for the study of β1C expression and role in prostate epithelial cell proliferation.
- Polyclonal antibodies to **β3** have been generated.
- PC3 stable cell lines expressing either **β1C** or **β1A** under the control of a tetracycline regulated promoter have been generated.
- **β3**-LNCaP and **β6**-LNCaP cell transfectants have been generated.
- **β3/6** chimera-LNCaP and **β6/3** chimera-LNCaP cell transfectants have been generated.

REPORTABLE OUTCOMES:

Manuscripts (*The contribution of the DAMD 17-98-1-8506 award has been acknowledged in the enclosed manuscripts*)

1.Zheng, D.Q., Woodard, A. S., Fornaro, M., Tallini, G. and **Languino, L.R.** Prostatic Carcinoma Cell Migration via $\alpha_V\beta_3$ integrin is modulated by a Focal Adhesion Kinase pathway. *Cancer Res.*, 59:1655-1664 (1999).

2.Fornaro, M. , Tallini, G., Zheng, D.Q., Flanagan, W.M., Manzotti, M., **Languino, L.R.** p27kip1 acts as a downstream effector of and is co-expressed with the β_{1C} integrin in prostatic adenocarcinoma. *J. Clin. Invest.*, 103:321-329 (1999).

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10.Fornaro, M., Plescia, J., Chheang, S., Tallini, G., Altieri, C.D., **Languino, L.R.** Fibronectin Adhesion Protects Prostate Cancer Cells from Tumor Necrosis Factor alpha-Induced Apoptosis via the AKT/Survivin Pathway. (2003, JBC, 2003, Submitted). [Reprints to follow when published].

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11. Woodard, A.S., G. García-Cardeña, M.Leong, J. A. Madri, W.C. Sessa and **Languino, L.R.** The synergistic activity of $\alpha_v\beta_3$ integrin and PDGF Receptor increases cell migration. *J. Cell Science* (1998) **111**:469-478.

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<u>Invited Reviews/Chapters</u> (*The contribution of the DAMD 17-98-1-8506 award has been acknowledged in the enclosed reviews/chapters*)

16.Steger, C.A., Fornaro, M., Zheng D.Q. and **Languino, L.R.** Expression of exogenous integrin subunits in mammalian cells. *In "Protocols for Adhesion Proteins-Methods in Molecular Biology". Humana Press,* 129:125-134 (1999).

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<u>Other relevant review by the PI:</u>
20.**Languino, L.R.** Cell adhesion :the outside and inside story In: "<u>*TRENDS in Cell Biology,*</u>" (2002), **12:**539 (Book Review).

<u>Abstracts</u>

21. **Languino, L.R**. Role of β1 integrins and β1 – downstream effectors in prostate cancer cell adhesion and proliferation. "*Prostate Cancer Research*" (2002). 2nd International Conference on Prostate Cancer Research: Bridging basic science with clinical medicine. Iowa City, Iowa. October 12-15 (2002).

22. Fornaro, M., Zheng, D.Q., Manzotti, M., Tallini, G., **Languino, L.R.** Regulation of Cancer Cell Proliferation and Survival by $\beta 1$ Integrins. *"Predictive Oncology and Intervention Strategies Conference"*. Paris, France February 9-12 (2002).

23. **Languino, L.R.** Moro,L., Manes, T., Fornaro, M., Zheng, D.Q., Adhesion Molecules. *"Tumor Biology"*. The XXX Meeting of the International Society for Oncodevelopmental Biology and Medicine, ISOBM Boston, MA. September 8-12 (2002).

24. Manes, T., Jain, D., Zheng, D.Q., Woodard, A.S., Tallini, G., and **Languino, L.R.** $\alpha_v\beta_3$, an integrin up-regulated in prostate cancer, increases cdc2 cyclin-dependent kinase levels. *"Experimental Biology"*. Orlando, FL, March 31-April 4 (2001).

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28.Fornaro M, Moro L., Bennett A.M. and **Languino, L.R.** Regulation of cell proliferation and apoptosis by β_1 integrin cytoplasmic variants. Gordon Conference on "Signaling by Adhesion Receptors", Salve Regina University, Newport, RI, July 23-28, 2000.

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30.Fornaro M, Tallini G, Zheng DQ, Flanagan W.M., Steger C.A. and **Languino, L.R.** The cyclin kinase inhibitor p27kip1 mediates the antiproliferative effect of β_{1C} integrin. Oncogene Network in Signal Transduction, Keystone, Colorado, April 9-14, 1999.

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32. **Languino, L.R.** Signaling by Integrins in Prostate Cancer. *"IBC's 2nd Annual Conference on Integrins"*. Boston, MA, March 18-19 (1999).

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33.Cohen M.B, Padarathsingh M., and Hendrix M.J.C. Experimental models of prostate cancer research. *Am. J. Pathol.* 156:355-358 (2000).

Invited Presentations

Speaker: Lucia R. Languino. "Prostate Cancer State of the Science Workshop" NCI, Washington, DC, November 14-15 (1999).

Speaker: Lucia R. Languino. "Fibronectins and Related Molecules" Gordon Conference, Ventura, CA, January 31-February 5 (1999).

Speaker: Mara Fornaro, in Languino's lab. "Signaling by Integrins in Prostate Cancer" IBC Conference, Boston, MA, March 18-19 (1999).

Speaker: Lucia R. Languino. "Wound Repair" Gordon Conference, New Hampshire, June 13-18 (1999).

Speaker: Lucia R. Languino. "Prostate Cancer" NIH Workshop, Iowa City, Iowa, June 24 (1999).

Speaker: Thomas Manes, in Languino's lab. Experimental Biology, Prostate Cancer Minisymposium, Orlando, FL, March 31-April 4 (2001).

Speaker: Lucia R. Languino. "Fibronectin and Related Molecules" Gordon Conference, Ventura, CA, February 4-9 (2001).

Speaker: Lucia R. Languino. "Tyrosine phosphorylation and cell signaling" Cold Spring Harbor, Long Island NY, May 16-20 (2001).

Speaker: Mara Fornaro, in Languino's lab. "Predictive Oncology and Intervention strategies" Paris, France, Feb 9-12 (2002),

Speaker: Lucia R. Languino. "Translational Cancer Research" ISOBM Conference, Boston, MA Sept. 8-12 (2002).

Speaker: Lucia R. Languino. "2nd International Conference on Prostate Cancer Research", Iowa City, Iowa, Oct. 12-15 (2002)

Panel member

"Prostate Cancer State of the Science Workshop"
NCI, Nov 14-15 (1999) Washington, DC

Development of Cell Lines

NRP152-β1C
NRP152-mock
CHO-β1C
CHO-β1A
CHO-mock
PC3-Ch1β1A
PC3-Chβ1C
PC3-β1C
PC3-β1A
PC3-mock
β3-LNCaP
β6-LNCaP
β3/6 chimera-LNCaP
β6/3 chimera-LNCaP
mock-LNCaP

Patent

A patent (US Patent No. 6,013,495) has been issued to Dr. Languino and her collaborators on the discovery of -β1C's ability to inhibit cell proliferation.

CONCLUSIONS

The interactions between cancer cells and extracellular matrix proteins are mediated by integrins, that have emerged as critical modulators of cell adhesion, proliferation, migration and intracellular signaling. The pathological consequences of integrin deregulated expression in cancer have been the focus of the PI's current investigations. The β1 and β3 integrins are differentially expressed in normal and neoplastic cells. Our recent findings show that their expression plays a pivotal role in modulating prostate cancer cell functions *in vivo*, since they significantly affect prostate tumor growth. Using genetically engineered and primary prostate cancer cells, we have found that, *in vitro*, the β1 and β3 integrins modulate cell adhesion and motility via activation of specific signaling events and via regulation of gene expression. Therefore, given their deregulated expression in neoplastic cells and their ability to control multiple downstream signals, the β1 and β3 integrins and their downstream effectors provide exciting opportunities for new approaches to cancer therapy.

"SO WHAT": Our recent publications pave the way for future investigations describing the role of integrin signaling in prostate cancer cell invasion, metastatic establishment and growth. Nowadays, significant controversies exist on which therapy constitutes the optimal treatment for prostate cancer. A better understanding of the biologic mechanisms responsible for the uncontrolled growth and motility of prostate cancer cells is critical to devise novel therapeutic approaches.

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APPENDICES

Final Report for Award Number DAMD 17-98-1-8506
Title: β1 and β3 Integrins in Prostate Cancer
Period: July 1998- December 2002
PI: Lucia R. Languino, Ph.D.

The contribution of the DAMD 17-98-1-8506 award has been acknowledged in all the enclosed papers.

Manuscripts

1.Fornaro, M., Lovecchio, M.R., Jose, P., Zheng, DQ., Moro, L., and **Languino, L.R**. Epitope-specific antibodies to the β1C integrin cytoplasmic domain variant. *Experimental and Molecular Pathology*, 70:275-280 (2001).

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5.Perlino, E., Lovecchio, M.R., Vacca, R.A., Fornaro, M., Moro, L., Ditonno, P., Battaglia, M., Selvaggi, F.P., Mastropasqua, M.G., Bufo, P., and **Languino, L.R.** Regulation of mRNA and protein levels of β1 integrin variants in human prostate carcinoma. *Am. J. Pathol.,* 157:1727-1734 (2000).

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7.Zheng, D.Q., Woodard, A. S., Fornaro, M., Tallini, G., and **Languino, L.R**. Prostatic Carcinoma Cell Migration via $\alpha_V\beta_3$ integrin is modulated by a Focal Adhesion Kinase pathway. *Cancer Res.*, 59:1655-1664 (1999).

<u>Invited Reviews/Chapters</u>

8.**Languino, L.R**. and Wells, R.G., Integrins. *In "The liver biology and pathobiology"*. Lippincott Williams & Wilkins, 33:475-482 (2001).

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<u>Abstracts</u>

11.**Languino, L.R**. Role of $\beta 1$ integrins and $\beta 1$ –downstream effectors in prostate cancer cell adhesion and proliferation. *"Prostate Cancer Research"* (2002). 2nd International Conference on Prostate Cancer Research: Bridging basic science with clinical medicine. Iowa City, Iowa. October 12-15 (2002).

12.**Languino, L.R.** Moro,L., Manes, T., Fornaro, M., Zheng, D.Q., Adhesion Molecules. *"Tumor Biology"*. The XXX Meeting of the International Society for Oncodevelopmental Biology and Medicine, ISOBM Boston, MA. September 8-12 (2002).

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15.Moro, L., Fornaro, M., Mccarthy., T.L., Centrella, M., **Languino, L.R.** A Novel Autocrine Mechanism Activated by $\beta 1$ Integrins that supports Cell Adhesion Via IGF-II and Type I IGF Receptor. *"Molecular Biology of the Cell"* Vol **12**:464a (2001).

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Epitope-Specific Antibodies to the β_{1C} Integrin Cytoplasmic Domain Variant

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The β_{1C} integrin is an alternatively spliced variant of the β_1 subunit that contains a unique 48-amino-acid sequence in its cytoplasmic domain. We have shown previously that β_{1C} is a potent inhibitor of cell proliferation and that *in vivo* its expression is downregulated in prostate and breast carcinoma. In this study, we describe a panel of specific monoclonal antibodies that react with the β_{1C} cytodomain. We show by immunoblot analysis that the newly generated monoclonal antibodies specifically recognize the β_{1C} cytodomain expressed as glutathione *S*-transferase fusion protein. The specificity of the antibodies to β_{1C} was confirmed in competition studies by immunoblotting using β_{1C}-specific synthetic peptides. These monoclonal antibodies reacted, in enzyme-linked immunosorbent assays, with the β_{1C} 785–808 peptide but failed to bind the β_{1C} 778–794, β_{1C} 805–825, or β_{1A} 765–798 peptides. Thus, the epitope recognized by the antibodies is located within the Q^{795}–F^{804} β_{1C} cytoplasmic sequence; this region overlaps the previously described Q^{795}–Q^{802} domain necessary for β_{1C} to inhibit cell proliferation. To our knowledge, these are the first monoclonal antibodies specific for a β_1 cytoplasmic isoform. The monoclonal antibodies described here will be useful tools for dissecting functional differences, among β_1 integrin variants, as well as for the study of the role of β_{1C} in prostate and breast epithelial cell proliferation.

Key Words: alternative spliced variants; integrin cytoplasmic domain; prostate cancer cells; breast cancer cells; cell proliferation.

INTRODUCTION

Integrins are a large family of transmembrane receptors composed of an α and a β subunit that have been shown to regulate cell growth, survival, and differentiation, in addition to cell adhesion to the extracellular matrix (Hynes, 1992; Ruoslahti and Reed, 1994).

It is well established that the cytoplasmic domain of the β subunit is required for integrins to modulate many cellular functions as well as to trigger signaling events that result in protein phosphorylation (Fornaro and Languino, 1997; Hemler *et al.*, 1995; Wei *et al.*, 1998) and interactions with intracellular proteins (Hemler, 1988). Therefore, structural differences in the amino acid sequence of the β subunit cytodomain can contribute to the specificity of integrin signaling.

The β_{1C} integrin is an alternatively spliced variant of the β_1 subfamily that contains a unique 48-amino-acid sequence in its cytoplasmic domain (Languino and Ruoslahti, 1992). The β_1 integrin subunits β_{1C} and β_{1A} that contain variant cytoplasmic domains differentially affect cell proliferation. It has been shown previously that either full-length β_{1C} or its cytoplasmic domain inhibits prostate cancer epithelial cell (Fornaro *et al.*, 1998; Meredith *et al.*, 1999), endothelial cell (Meredith *et al.*, 1999), and fibroblast (Fornaro *et al.*, 1995; Meredith *et al.*, 1995, 1999) proliferation, whereas β_{1A} promotes it. *In vivo*, β_{1C} is expressed in nonproliferative,

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differentiated epithelium; its expression is selectively downregulated in prostatic adenocarcinoma and inversely correlates with markers of cell proliferation in breast carcinoma (Fornaro *et al.*, 1996, 1998, 1999; Manzotti *et al.*, 2000). Moreover, in a recent study we elucidated β_{1C}-mediated downstream signaling pathways that control cell proliferation and survival. Specifically, we have shown that at variance with β_{1A}, β_{1C} strongly inhibits cell proliferation by preventing Ras/MAP kinase pathway activation (Fornaro *et al.*, 2000). By deletion analysis, we reported earlier an 8-amino acid region (Q^{795}–Q^{802}) within the β_{1C} cytoplasmic domain that is necessary and sufficient to inhibit cell proliferation (Fornaro *et al.*, 1995).

We describe here the characterization of a panel of monoclonal antibodies (mAbs) that specifically recognize the β_{1C} Q^{795}–F^{804} sequence. To our knowledge, these are the first mAbs specific for a β_1 cytoplasmic isoform. These epitope-specific antibodies will be useful for dissecting functional differences among β_1 integrin variants and will help in elucidating β_{1C} downstream effectors and their role in prostate and breast epithelial cell proliferation.

MATERIALS AND METHODS

Reagents and Antibodies

Rabbit antibodies specific for the β_{1C} subunit cytoplasmic domain were affinity-purified as described previously (Fornaro *et al.*, 1996). The following antibodies were used: mouse mAb K20 to human β_1 integrin (Coulter–Immunotech, Miami, FL); rabbit affinity-purified antibody to ERK1 and 2 (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit antiserum to β_{1A} integrin (Chemicon International, Inc., Temecula, CA); goat affinity-purified antibody to glutathione *S*-transferase (GST; Amersham Pharmacia Biotech Inc., Piscataway, NJ). Nonimmune mouse Ig (mIg) was purchased from Sigma Chemical Co. (St. Louis, MO). β_{1C}- and β_{1A}-specific synthetic peptides used for this study were β_{1C} 778–794 (kkSLSVAQPGVQWCDISSL), β_{1C} 785–808 (kkVQWCDISSLQLTSRFQQFSCLS), β_{1C} 805–825 (kkSCLSLPSTWDYRVKILFIRVP), β_{1C} 795–812 (kkQPLTSRFQQFSCLSLPST), β_{1C} 778–825 (kkSLSVAQPGVQWCDISSLQLTSRFQQFSCLSLPSTWDYRVKILFIRVP), and β_{1A} 765–798 (KFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK). A peptide derived from the β_3 integrin cytoplasmic domain sequence [762–788 (RAKWDTANNPLYKEATSTFTNITYRGT)] was used as a control in enzyme-linked immunosorbent assay (ELISA) and immunoblotting analysis.

Generation of β_{1C}-Specific mAbs

The β_{1C}-specific mAbs were generated in BALB/c mice (Cocalico Biologicals Inc., Reamstown, PA) using, as immunogens, synthetic peptides [778–825 (kkSLSVAQPGVQWCDISSLQLTSRFQQFSCLSLPSTWDYRVKILFIRVP) and 795–812 (kkQPLTSRFQQFSCLSLPST)] from the deduced sequence of β_{1C} (Languino and Ruoslahti, 1992).

Plasmids and Recombinant Proteins

The GST–β_{1C} fusion protein consisting of amino acids 778–825 of the human β_{1C}-specific cytodomain fused to GST was generated as follows: a 146-bp cDNA fragment corresponding to nucleotides 2435 to 2581 from the cytoplasmic domain of human β_{1C} was generated by polymerase chain reaction (PCR) as described (Languino and Ruoslahti, 1992). Primers contained *Bam*HI (5′-CG<u>GGATC</u>CCTCTCTCTCTGTCGC-3′) and *Eco*RI (5′-CG<u>GAATTC</u>TTACGGCACTCTT-3′) sites at their 5′ ends (restriction sites are underlined). The amplified product was subcloned into *Bam*HI and *Eco*RI sites in the pGEX–2T plasmid (Amersham Pharmacia Biotech Inc.). The fragment generated by PCR was sequenced by the dideoxy chain-termination reaction using Sequenase 2.0 (United States Biochemical, Cleveland, OH). Either GST or the GST–β_{1C} fusion protein expression in transformed JM105 *Escherichia coli* was induced using 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (American Bioanalytical, Natick, MA) for 1 h at 37°C. Fusion proteins were isolated by affinity chromatography on glutathione–Sepharose 4B according to the manufacturer's instructions (Amersham Pharmacia Biotech Inc.).

Immunoblotting

Ten micrograms of purified GST or GST–β_{1C} fusion proteins were separated by 15% SDS–PAGE and transferred to PVDF membranes (Millipore, Bedford, MA) as described previously (Fornaro *et al.*, 1996). Membranes were then blocked in Tris-buffered saline (TBS; 20 mM Tris, pH 7.5, 150 mM NaCl) containing 0.3% Tween 20 (American Bioanalytical) and 5% nonfat dry milk for 3 h at room temperature. Immunostaining was performed using either a mAb to β_{1C} or a control culture supernatant (1:10 dilution of culture supernatant in blocking buffer) for 1 h at room temperature. Membranes were then washed three times in TBS containing

0.3% Tween 20 (TBS-T) and incubated with horseradish peroxidase-conjugated affinity-purified antibody to mouse Ig (Amersham Pharmacia Biotech Inc.) in blocking buffer for 1 h at room temperature. After three washes in TBS-T, proteins were visualized using the enhanced chemiluminescence system (ECL; NEN, Boston, MA). Goat affinity-purified antibody to GST (5 μg/ml) was used to control for protein loading. The specificity of the mAb reactivity for β_{1C} was confirmed for mAb BA6 in immunoblotting by incubating the BA6 culture supernatant with 30 μg/ml of either a mixture of three β_{1C}-specific peptides (β_{1C} 778–794, β_{1C} 785–808, and β_{1C} 805–825) or a negative control β_3-derived peptide.

ELISA

Epitope mapping of the mAbs to β_{1C} was performed by ELISA as described previously (Fornaro *et al.*, 1996) using 96-well microtiter ELISA plates coated with 20 μg/ml synthetic peptides containing β_{1C} cytoplasmic domain sequences. A synthetic β_{1A}- or a β_3-derived peptide was used as control.

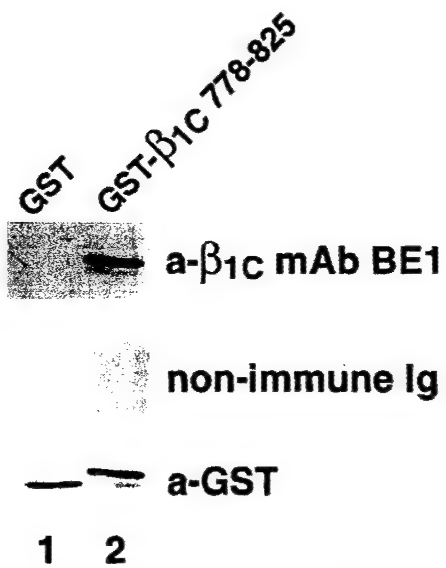

FIG. 1. Characterization of the newly generated mAbs to β_{1C} by immunoblot analysis. A representative mAb is shown. Ten micrograms of either GST (lane 1) or GST–β_{1C} (lane 2) fusion protein was separated by 15% SDS-PAGE and transferred to PVDF membranes. The GST–β_{1C} fusion protein was detected by immunoblotting using either BE1, mAb to β_{1C}, or nonimmune Ig as a negative control (1:10 dilution of culture supernatant; top and middle panels, respectively). Membranes were reblotted using 5 μg/ml affinity-purified antibody to GST (bottom panel) to detect GST-containing proteins. Proteins were visualized by ECL.

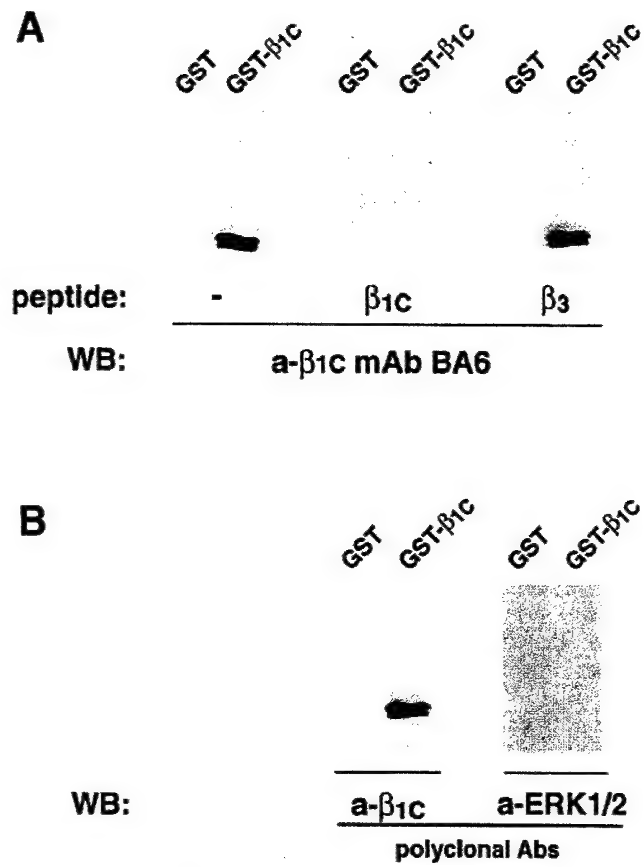

FIG. 2. MAb specificity for the β_{1C} cytodomain. (A and B) Ten micrograms of either GST or GST–β_{1C} fusion protein was separated by 15% SDS-PAGE and transferred to PVDF membranes. (A) The GST–β_{1C} fusion protein was detected by immunoblotting using BA6, mAb to β_{1C} (1:10 dilution of culture supernatant; left panel). β_{1C} immunostaining was specifically inhibited by incubating BA6 with 30 μg/ml of a mixture of three β_{1C}-specific peptides containing overlapping sequences of the β_{1C} cytoplasmic domain (β_{1C} 778–794, β_{1C} 785–808, and β_{1C} 805–825; middle panel) but not with 30 μg/ml of a control β_3-derived peptide (right panel). (B) Membranes used for the competition study described for A were reblotted with 0.1 μg/ml affinity-purified polyclonal antibody either to β_{1C} (left panel) or to ERK1/2 as a negative control (right panel). Proteins were visualized by ECL. WB, Western blotting.

RESULTS AND DISCUSSION

Antibody Specificity

β_{1C}-specific mAbs were generated in BALB/c mice immunized with a mixture of two synthetic peptides containing overlapping sequences in the β_{1C} 778–825 cytoplasmic domain. The immunoreactivity of the mAbs to the β_{1C} cytoplasmic domain was assessed by immunoblotting using a

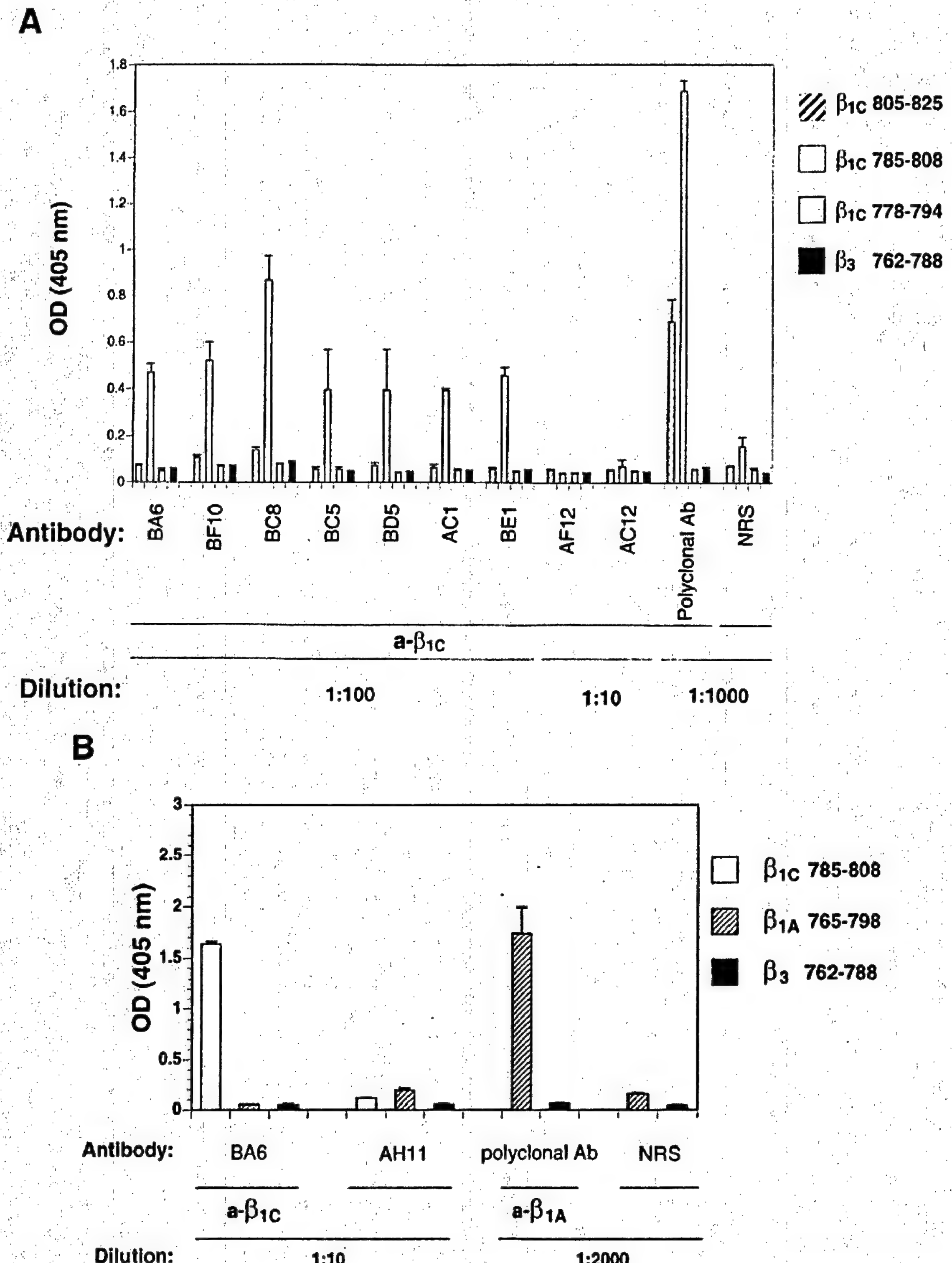

FIG. 3. Epitope mapping of mAbs to β_{1C}. Ninety-six-well microtiter ELISA plates were coated with 20 μg/ml synthetic peptides containing either β_{1C} or β_3 (A and B) or β_{1A} (B) cytoplasmic domain sequences. The dilution of the mAbs used (BA6, mAb to β_{1C}, and AH11, negative control Ab) was either 1:100 or 1:10. In A, rabbit antiserum to β_{1C} (polyclonal Ab) and normal rabbit serum (NRS) were used at 1:1000 dilution. In B, rabbit antiserum to β_{1A} (polyclonal Ab) and NRS were used at 1:2000 dilution. Absorbance was quantitated at 405 nm.

778 795 804 825

SLSVAQPGVQWCDISSL[QPLTSRFQQF]SCLSLPSTWDYRVKILFIRVP

{ BA6, BF10, BC8, BC5, BD5, AC1, BE1 }

FIG. 4. The amino acid sequence of the epitope recognized by the newly generated mAbs to β_{1C}. The epitope mapping results shown in this study indicate that the Q^{795}–F^{804} amino acid sequence (box) within the β_{1C} cytoplasmic domain is recognized by the newly generated mAbs. The 8-amino-acid region (Q^{795}–Q^{802}) shown to be necessary and sufficient to inhibit cell proliferation (Fornaro *et al.*, 1995) is in bold. The mAbs that recognize the β_{1C} Q^{795}–F^{804} amino acid sequence are indicated between brackets below the epitope sequence.

GST recombinant protein containing the β_{1C} S^{778}–P^{825} sequence. Seven mAbs (BA6, BF10, BC8, BC5, BD5, AC1, and BE1) that specifically recognized the GST–β_{1C} fusion protein but not GST alone (Fig. 1 and not shown) were selected for further characterization.

The specificity of the reactivity of the mAbs to β_{1C} was confirmed by immunoblotting analysis in competition studies using β_{1C}-specific peptides. As shown in Fig. 2A, incubation of BA6, mAb to β_{1C}, with a mixture of three β_{1C}-specific peptides (β_{1C} 778–794, β_{1C} 785–808, and β_{1C} 805–825), but not with a control β_3-derived peptide, inhibited BA6 reactivity with the GST–β_{1C} fusion protein. Membranes used for competition studies were reblotted with affinity-purified polyclonal antibody to β_{1C} to control for GST–β_{1C} fusion protein loading (Fig. 2B). Three of the seven selected mAbs (BA6, BF10, and BC8) specifically recognized in immunoblotting the human native β_{1C} immunoprecipitated from detergent extracts generated from CHO-β_{1C} stable cell transfectants (Fornaro *et al.*, 2000) using K20, mAb to human β_1 integrin (data not shown). The results show that the newly generated mAbs specifically recognize β_{1C} cytoplasmic domain sequences.

Epitope Mapping

In ELISA, the seven selected mAbs recognized the G^{785}–S^{808} β_{1C} sequence but failed to react with two different β_{1C}-specific peptides (S^{778}–L^{794} and S^{805}–P^{825}), with a control β_3-derived peptide (Fig. 3A), or with a synthetic peptide containing β_{1A} cytoplasmic domain sequences (Fig. 3B). Therefore, the newly generated mAbs recognize a specific epitope (Q^{795}–F^{804}) within the β_{1C} cytodomain (Fig. 4).

In conclusion, we have generated a panel of mAbs that recognize the Q^{795}–F^{804} sequence within the β_{1C} cytoplasmic domain. This region overlaps the previously described Q^{795}–Q^{802} domain in β_{1C} that inhibits cell proliferation (Fig. 4). These epitope-specific antibodies will be used to study both β_{1C}-specific interactions with intracellular proteins and β_{1C} downstream signaling pathways, thus bringing new insights into the role of β_1 integrins in prostate and breast epithelial cell proliferation. Furthermore, these mAbs will be a useful tool for studying the expression of specific β_1 integrin variants in human benign and malignant tissues.

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Regulation of MCP-3 and BRCA2 mRNA Expression Levels by β_1 Integrins

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The integrin cytoplasmic domain has been shown to modulate several cellular functions, including cell proliferation, adhesion, migration, and intracellular signaling. The β_1 integrin subunits β_{1C} and β_{1A}, which contain variant cytoplasmic domains, differentially affect cancer and normal cell functions. To identify target genes selectively regulated by these β_1 cytoplasmic variants, stable cell transfectants expressing either β_{1A} or β_{1C} under the control of a doxycycline-inducible promoter were obtained using murine β_1-deficient GD25 cells. Screening of 1176 murine cDNAs using first-strand cDNA of mRNA isolated from either β_{1C}- or β_{1A}-expressing cells showed a striking differential expression of few genes. The differential expression of two genes, MCP-3 and BRCA2 (monocyte chemoattractant protein-3 and breast cancer susceptibility gene 2, respectively), whose products are involved, respectively, in chemotaxis and embryonic proliferation, was confirmed by Northern blot analysis. Increased MCP-3 and decreased BRCA2 mRNA levels in cells expressing β_{1C} compared to those in cells expressing β_{1A} were observed. Since β_{1C} and β_{1A} stable cell transfectants showed comparable adhesion to fibronectin, upregulation of MCP-3 and downregulation of BRCA2 mRNA levels did not appear to be due to a differential ability of the β_{1C} cells to adhere to the β_1 ligand fibronectin. Overall, our data show that β_1 integrin cytoplasmic domain variants control expression of downstream target genes in a differential manner without affecting cell adhesion. © 2001 Academic Press

Key Words: integrin; cytoplasmic domain; gene expression; adhesion; fibronectin; vitronectin.

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INTRODUCTION

Integrins have emerged as modulators of a variety of cellular functions (Ruoslahti and Reed, 1994). They have been implicated in organ and tissue development, normal and aberrant cellular growth, and modulation of intracellular signal transduction mechanisms (Damsky and Werb, 1992; Haas and Madri, 1999; Hynes, 1999; Juliano, 1996). Integrins are structurally organized into heterodimeric transmembrane complexes, variously assembled through the noncovalent association between an α and a β subunit (Hemler *et al.*, 1995). So far, 18 α subunits, 8 β subunits, and 22 complexes have been identified and their expression and function characterized in various cell types. The integrin family is divided into subfamilies that share the β subunit (Ruoslahti, 1997). Each β subunit associates with 1 to 8 α subunits and each α can associate with more than one β subunit. Functional specificity is determined by the associated subunits and by the cell type that expresses the heterodimeric complex.

Each subunit has a large extracellular domain, a single transmembrane domain, and a short cytoplasmic domain. The role of the integrin cytoplasmic domain in modulating integrin functions and signaling events is well established. Recent experimental evidence obtained with recombinant deletion mutants and chimeric forms of α and β integrin cytoplasmic domains has demonstrated that cytoplasmic tails modulate receptor distribution, receptor surface expression,

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ligand binding affinity of the extracellular domain, cell adhesion, and cell spreading (Fornaro and Languino, 1997; Hemler *et al.*, 1995). Therefore, structural differences in the primary sequences of the integrin intracellular domains are predicted to determine the specificity of a variety of integrin-mediated events. In support of this hypothesis, mutations and deletions in the integrin cytoplasmic domain have been found in the β_3 and β_4 integrin subgroups in, Glanzmann's thrombasthenia (Williams *et al.*, 1994) and junctional epidermolysis bullosa (Vidal *et al.*, 1995), respectively, thus pointing to the cytoplasmic domain as a key player in determining crucial cellular responses *in vivo*.

Alternatively spliced forms of β and α integrin cytoplasmic domains have been identified [for reviews see Hemler *et al.* (1995) and Fornaro and Languino (1997)], thus adding further complexity to the regulatory pathways mediated by integrins. Five different β_1 isoforms containing alternatively spliced cytoplasmic domains have been identified (β_{1A}, β_{1B}, β_{1C}, β_{1C-2}, and β_{1D}) and have been shown to differentially affect receptor localization, cell proliferation, cell adhesion and migration, interactions with intracellular proteins, and, ultimately, phosphorylation and activation of signaling molecules (Fornaro and Languino, 1997; Svineng *et al.*, 1998; Fornaro *et al.*, 2000). Specifically, β_{1C} and β_{1A} differentially affect cell proliferation (Fornaro *et al.*, 2000).

Cell adhesion to extracellular matrix (ECM) proteins via β_1 integrins but not integrin expression per se has been shown to upregulate mRNA levels of metalloproteinases (Romanic and Madri, 1994; Huhtala *et al.*, 1995; Werb *et al.*, 1989) and transcription factors [reviewed in Juliano and Haskill (1993)]. Furthermore, a differential role for β_1 cytoplasmic domain variants in modulating gene expression has not been studied. We show here increased MCP-3 [monocyte chemoattractant protein-3; Haelens *et al.* (1996), Fioretti *et al.* (1998)] and decreased BRCA2 [breast cancer susceptibility gene 2; Welcsh *et al.* (2000)] mRNA levels in cells expressing β_{1C} compared to cells expressing β_{1A}. Since β_{1C}- and β_{1A}-expressing cells showed comparable adhesion to fibronectin, upregulation of MCP-3 and downregulation of BRCA2 mRNA levels do not appear to be due to a differential ability of these cells to adhere to the β_1 ligand fibronectin. Overall, our data show that integrin cytoplasmic domain variants modulate the expression of downstream target genes in a differential manner without affecting cell adhesion.

MATERIALS AND METHODS

Reagents and Antibodies

The following antibodies were used: mouse monoclonal antibody (mAb) TS2/16 to human β_1 integrin (ATCC, Rockville, MD); mAb 12CA5 to hemagglutinin (ATCC); mAb 1C10 to a vascular endothelial surface protein (Life Technologies, Gaithersburg, MD); hamster mAb H9.2B8 to mouse α_v (PharMingen, San Diego, CA); mAb GoH3 to α_6 (provided by Dr. Sonnenberg, The Netherlands Cancer Institute, Amsterdam); and rabbit antisera to β_3, α_2, or α_v cytoplasmic domains. Human plasma fibronectin and human vitronectin were purified as described (Engvall and Ruoslahti, 1977; Yatohgo *et al.*, 1988).

Cell Lines and Transfectants

The GD25 cell line, derived from β_1-deficient embryonic stem cells (Fassler *et al.*, 1995; Wennerberg *et al.*, 1996), was a kind gift of Dr. Fässler (Department of Experimental Pathology, Lund University, S-22285 Lund, Sweden). GD25 cells were cultured in DME medium (Life Technologies) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 0.292 μg/ml glutamine (all from Gemini Bio-Products, Inc., Calabasas, CA) and 250 μg/ml amphotericin B (Sigma Chemical Co., St. Louis, MO). GD25 stable cell transfectants expressing human β_{1A} or β_{1C} were obtained as follows: GD25 cells were plated at 80,000 cells/60-mm plate and, after 24 h, transfected using lipofectin (Life Technologies) according to the manufacturer's instructions with either 1 μg pTet–β_{1A} or 1 μg pTet–β_{1C} expression constructs (Fornaro *et al.*, 1999, 2000). In addition, 1 μg pTet–(r)tTA (Clontech, Palo Alto, CA) and 0.1 μg pTet–tTS vector (Clontech) encoding the doxycycline-controlled transcriptional silencer (to prevent doxycycline-independent gene expression) and 0.1 μg pTK–Hyg vector (Clontech) (to select for hygromycin resistance) were added to the lipofectin mixture. Hygromycin-resistant cells were selected using growth medium containing 150 μg/ml hygromycin (Boehringer Mannheim, Mannheim, Germany), and clones were screened for cell surface expression of β_{1A} and β_{1C} integrins by FACS using TS2/16, mAb against human β_1 integrin, or 12CA5 mAb as a negative control, as described (Fornaro *et al.*, 1995). To obtain clones expressing higher levels of β_{1A} and β_{1C} integrins, cells were sorted by FACS using TS2/16 mAb. After cell sorting, the stable transfectants were maintained in growth medium supplemented with 150 μg/ml hygromycin.

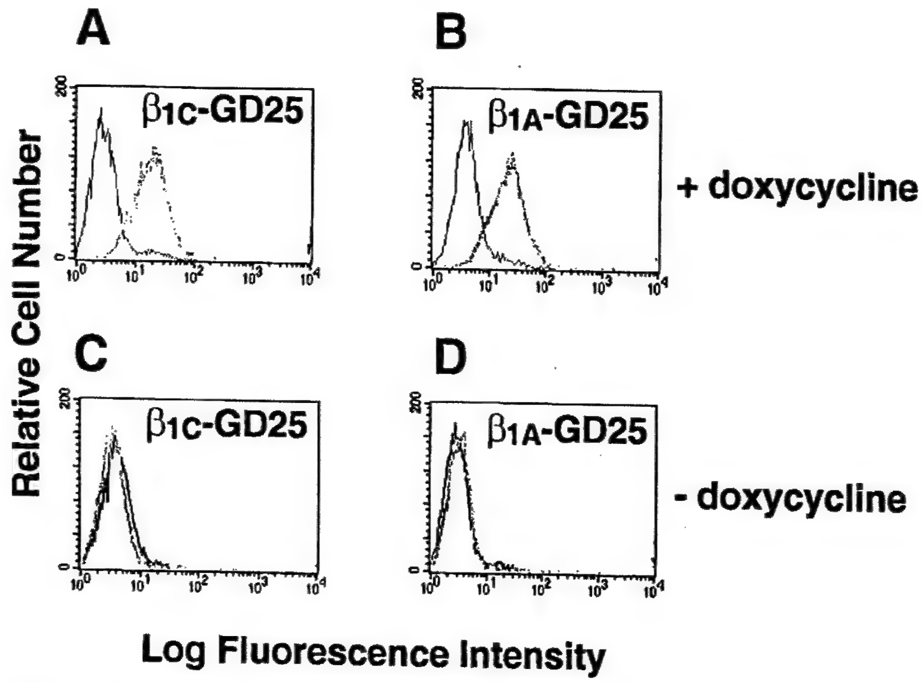

FIG. 1. Doxycycline-inducible expression of β_{1C} and β_{1A} in GD25 stable transfectants. (A–D) β_{1C}- and β_{1A}-GD25 stable cell lines were cultured for 24 h either in the presence (A, B) or in the absence (C, D) of 2 μg/ml doxycycline and analyzed by FACS using TS2/16, mAb to human β_1 integrin, or 12CA5 as a negative control, followed by FITC–goat anti-mouse IgG. Fluorescence intensity is expressed in arbitrary units. FACS analysis of a representative clone for each β_1 variant is shown. *Gray line*, TS2/16; *black line*, 12CA5.

CHO stable cell transfectants expressing either human β_{1A} or β_{1C} (Fornaro *et al.*, 2000) were cultured in DME medium supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.292 μg/ml glutamine, 0.1 mM nonessential amino acids (Life Technologies), 1 μg/ml tetracycline (Boehringer Mannheim), and 0.1 mg/ml G418 (Life Technologies).

Flow Cytometry

Surface expression of exogenous human β_{1C} or β_{1A} integrins in GD25 transfectants was achieved by addition of 2 μg/ml doxycycline (Clontech) in the growth medium; in both cell transfectants, β_{1C} or β_{1A} expression was maximal 24 h after doxycycline addition and was comparable in all the analyzed β_{1C} and β_{1A} clones. In each experiment, exogenous human β_1 integrin expression was monitored in GD25 cells by FACS using TS2/16 culture supernatant or 12CA5 as a negative control antibody (Fornaro *et al.*, 1999).

Immunoprecipitation of α Subunits Associated with β Integrins

β_1-GD25 stable transfectants were cultured for 24 h in the presence of 2 μg/ml doxycycline. β_1-null GD25 cells were cultured for 24 h in the absence of doxycycline. Cells were detached with 0.05% trypsin/0.53 mM EDTA and surface iodinated as described previously (Bartfeld *et al.*, 1993). Cells were lysed in 1% Triton X-100, 150 mM NaCl, 20 mM Tris, pH 7.5, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 μM calpain inhibitor, 1 mM $MgCl_2$, and 1 mM $CaCl_2$. β_1 and β_3 integrins were immunoprecipitated with TS2/16 and polyclonal Ab to β_3, respectively, and protein A-Sepharose (Sigma) as described (Fornaro *et al.*, 1995). Immunoprecipitates were washed five times with lysis buffer, resuspended in 10 mM Tris–HCl, pH 7.5, 0.5% sodium dodecyl sulfate (SDS), and incubated for 10 min at 70°C. The eluted material was diluted threefold with lysis buffer and reprecipitated with rabbit antiserum to α_2, α_v, and mAb to α_6 overnight at 4°C. Immunoprecipitates were recovered with protein A-Sepharose, washed three times with lysis buffer, and resuspended in loading buffer.

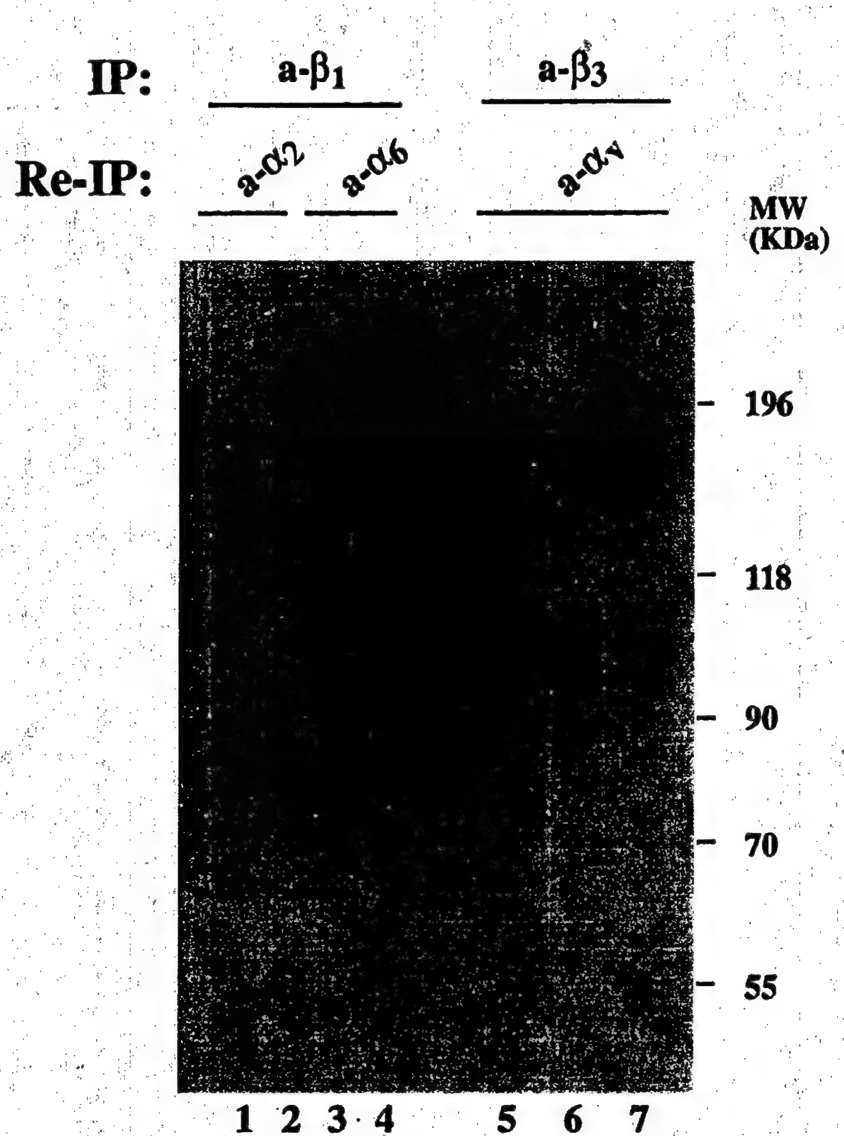

FIG. 2. Association of β_{1C} and β_{1A} with the α_6 subunit. β_{1C} and β_{1A}-GD25 stable cell transfectants were cultured for 24 h in the presence of 2 μg/ml doxycycline. β_1-null GD25 cells were kept in the same conditions in the absence of doxycycline. Cells were surface iodinated, lysed, and exogenous β_1 and endogenous β_3 integrins were immunoprecipitated using either TS2/16, mAb to β_1, or rabbit antiserum to β_3, respectively. The immunoprecipitated material was then eluted from protein A-Sepharose with 10 mM Tris–HCl, pH 7.5, 0.5% SDS for 10 min at 70°C, reprecipitated with rabbit antiserum to α_2 (lanes 1 and 2), mAb to α_6 (lanes 3 and 4), or, as control, rabbit antiserum to α_v (lanes 5–7), and separated by 7.5% SDS–PAGE. Lanes 1, 3, 5, β_{1C}-GD25; lanes 2, 4, 7, β_{1A}-GD25; lane 6, β_1-null GD25. Proteins were detected by autoradiography. Prestained molecular weight markers in kilodaltons (kDa) are shown.

Proteins were separated by SDS–PAGE (7.5%) and visualized by autoradiography.

Cell Adhesion Assay

Cell adhesion to fibronectin (5 μg/ml), vitronectin (0.3 μg/ml), and bovine serum albumin (BSA, 10 mg/ml; Sigma) was performed as previously described (Languino *et al.*, 1993) by incubating 25,000 ^{51}Cr-labeled (DuPont NEN, Wilmington, DE) cells with the coated substrates for 3 h at 37°C. Then, the attached cells were washed three times with PBS (pH 7.4; 137 mM NaCl, 2.7 mM KCl, 1 mM Na_2HPO_4, and 1.8 mM KH_2PO_4), lysed with 10% SDS, and counted in a scintillation counter (LS 6500 Beckman

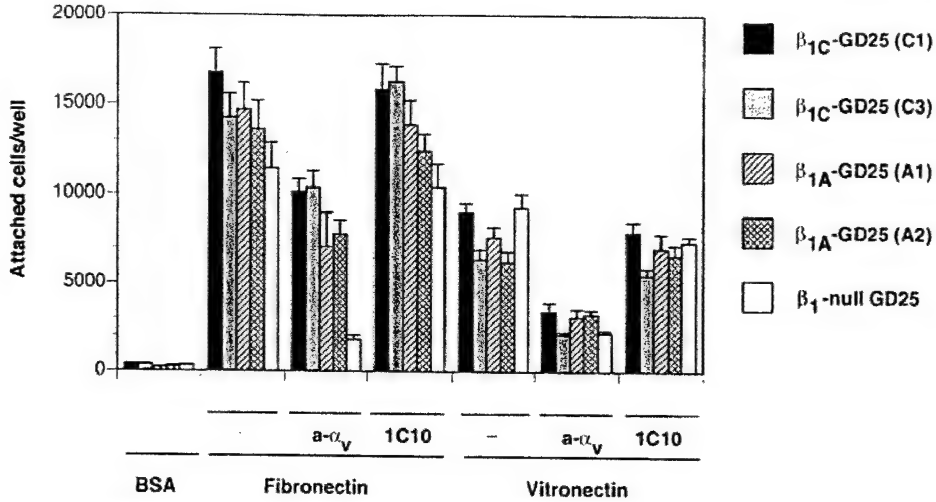

FIG. 3. β_{1C}- and β_{1A}-GD25 cell adhesion to fibronectin and vitronectin. β_{1C}-GD25 (clones: C1, C3), β_{1A}-GD25 (clones: A1, A2), and β_1-null GD25 cells were cultured for 24 h either in the presence (β_{1C}- and β_{1A}-GD25 clones) or in the absence (β_1-null GD25) of 2 μg/ml doxycycline. 2.5×10^4 cells were labeled using ^{51}Cr-sodium chromate, incubated for 1 h on ice in the presence or in the absence of mAb to murine α_v integrin (clone H9.2B8, ascites, 1:200) or 1C10 (1:200) as a negative control, and then allowed to attach to BSA (10 mg/ml), fibronectin (5 μg/ml), or vitronectin (0.3 μg/ml) at 37°C for 3 h. Data are expressed as means ± SD (n = 3).

Instruments, Columbia, MD). Inhibition assays were performed by incubating ^{51}Cr-labeled cells for 1 h on ice in the presence of either H9.2B8 mAb to murine α_v integrin (ascites 1:200) or 1C10 (ascites 1:200) as a negative control.

Migration Assay

100,000 GD25 or 50,000 CHO stable cell transfectants were resuspended in DME medium containing 0.2% BSA and plated in transwell migration chambers (8-μm pores for CHO and 5-μm pores for GD25; Corning Costar Corporation, Cambridge, MA) as described (Woodard *et al.*, 1998). Cells were allowed to migrate for 6 h at 37°C in the presence of 7.5% CO_2, fixed using 3% paraformaldehyde, and subsequently stained with 0.5% (CHO cells) or 1% (GD25 cells) Toluidine blue (Sigma) at room temperature. Cells that had not migrated were removed by wiping the top of the membrane with a cotton swab. The stained cells in 10 randomly chosen fields per filter were counted by microscopic examination and reported as numbers of migrated cells/mm^2.

RNA Isolation and Analysis

Gene expression profiles of β_{1A} or β_{1C} cell transfectants were generated using 1.2 Atlas Mouse cDNA Expression Arrays (Clontech) according to the manufacturer's instructions. GD25 stable cell transfectants were starved for 48 h. During the last 24 h, cells were kept in the presence of 2 μg/ml doxycycline and then detached using 0.05% trypsin/0.53 mM EDTA. Cells were washed three times with serum-free medium and plated ($3–5 \times 10^6$ cells/plate) on 150-mm plates coated with 5 μg/ml fibronectin for 5 h at 37°C. Attached cells were cultured for 8 h at 37°C in growth medium containing 10% FCS in the presence of 2 μg/ml doxycycline, trypsinized, and washed three times with PBS. mRNA was isolated and labeled with [α-^{32}P]dATP (Amersham Pharmacia Biotech, Piscataway, NJ) using the Atlas Pure Total RNA Labeling System (Clontech) according to the manufacturer's instructions. Following hybridization and washing per the manufacturer's instructions, arrays were visualized and quantitated using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Northern blot analysis was performed using total RNA isolated from cells kept in the same conditions as described above for the cDNA expression array analysis, cultured either in the presence or in the absence of 2 μg/ml doxycycline and in the presence of 10% FCS for 8 h upon adhesion to fibronectin. Total RNA (10 μg), isolated using TRIzol Reagent (Life Technologies), was electrophoresed through 1.5% denaturing agarose gel containing 660 mmol/L formaldehyde and transferred to a nylon membrane (Hybond N$^+$, Amersham Pharmacia Biotech). The filters were subsequently prehybridized for 4 h at 42°C with a buffer consisting

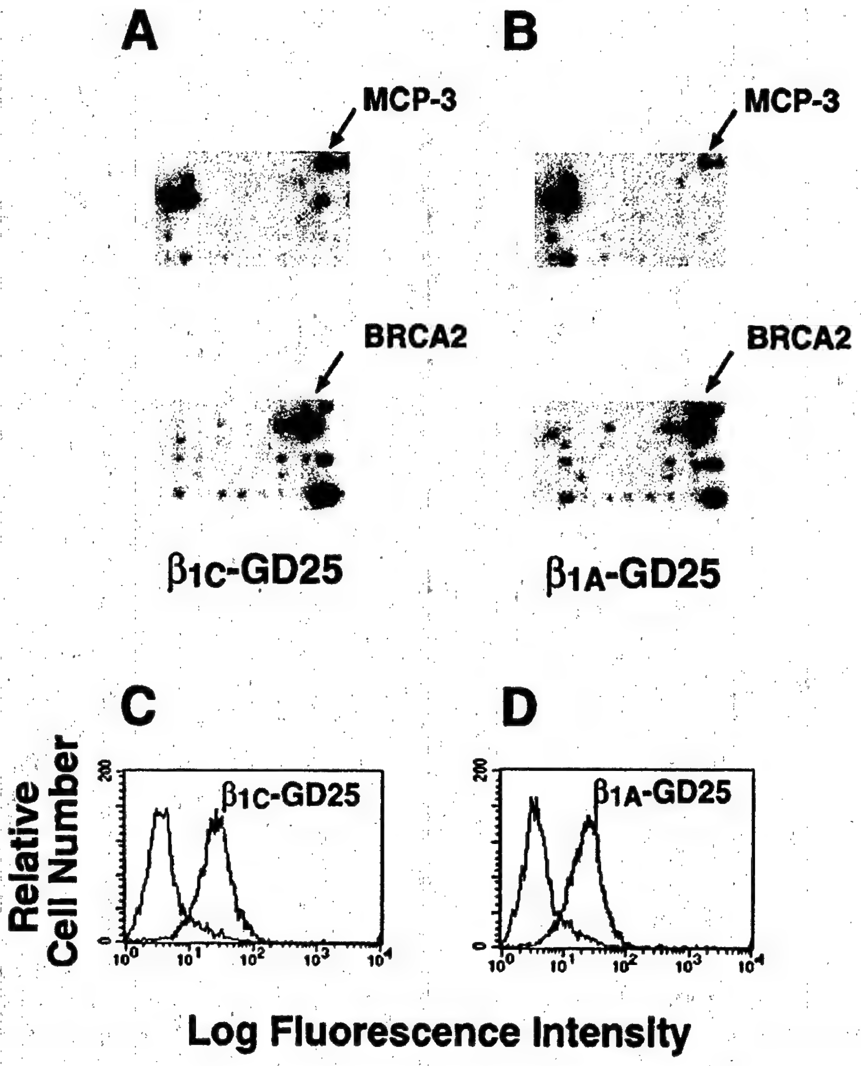

FIG. 4. cDNA expression array analysis in β_{1C}- and β_{1A}-GD25 cells. (A–D) GD25 stable cell transfectants were starved for 48 h. During the last 24 h, cells were kept in the presence of 2 μg/ml doxycycline and then detached, resuspended in serum-free medium, and plated on fibronectin (5 μg/ml) for 5 h. Attached cells were cultured for an additional 8 h in the presence of 10% FCS and 2 μg/ml doxycycline, then detached and processed for cDNA expression array (A, B) or FACS analysis (C, D). ^{32}P-labeled first-strand cDNA probes prepared with a mixture of equal amounts of mRNAs isolated from three β_{1C}-GD25 clones (C1, C2, C3) (A) and three β_{1A}-GD25 clones (A1, A2, A3) (B) were hybridized to 1.2 Atlas Mouse cDNA expression arrays. Shown are sections D (for MCP-3) and C (for BRCA2) of one cDNA array membrane. Similar results were obtained with another pair of array membranes hybridized using the same ^{32}P-labeled cDNA in the same experiment (not shown). In A and B, the arrows indicate the spots corresponding to MCP-3 or BRCA2 cDNA on the array. FACS analysis of β_1 integrin surface expression in β_{1C}-GD25 (C) and β_{1A}-GD25 (D) cells used for cDNA array screening was performed as described in the legend to Fig. 1. FACS analysis of a representative clone for each β_1 variant is shown. *Gray line*, TS2/16; *black line*, 12CA5.

of 50% formamide, 5× Denhardt's solution (1% Ficoll 400, 1% Polyvinylpyrrolidone, 1% BSA), 5× SSC (3 mol/L NaCl, 200 mmol/L NaH_2PO_4, pH 7.0, 19 mmol/L EDTA), 0.5% SDS, and 100 μg/ml sonicated salmon sperm DNA. The filters were then hybridized for 16 to 20 h at 42°C by adding 3–4 × 10^6 cpm of ^{32}P-labeled probe/ml to the prehybridization solution. The filters were washed once with 2× SSC, 0.1% SDS for 10 min at room temperature and then with 1× SSC, 0.1% SDS at 42°C, followed by several washes in 0.2× SSC, 0.1% SDS at 55°C. Bands were visualized by exposing the filters in a Phosphorimager and/or by autoradiography. The MCP-3 probe was a 0.8-kb *Eco*RI/*Xho*I restriction fragment excised from a murine MCP-3 cDNA clone [kindly provided by Dr. A. Mantovani, Istituto

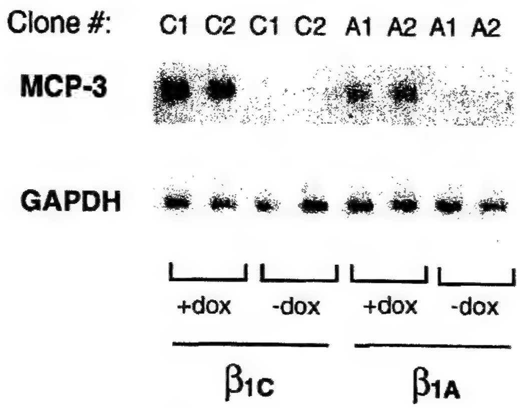

FIG. 5. Increased MCP-3 mRNA levels in β_{1C}-GD25 cells. Total RNA (10 μg) was isolated from β_{1C}-GD25 cells (clones: C1, C2) and β_{1A}-GD25 cells (clones: A1, A2) grown as described in the legend to Fig. 4, either in the presence or in the absence of 2 μg/ml doxycycline. Total RNA was fractionated by agarose–formaldehyde gel electrophoresis, transferred to Hybond N+ membranes, and hybridized using a 0.8-kb MCP-3 cDNA probe. GAPDH cDNA was used as a probe to control for RNA loading.

Ricerche Farmacologiche Mario Negri, Milan, Italy; Haelens *et al.* (1996)] and the BRCA2 probe was a 5.7-kb *NotI*/*XhoI* restriction fragment containing 4.3 kb of exon 11 and 1.4 kb of the preceding intron [kindly provided by Dr. A. Ashworth, Institute for Cancer Research, London, UK; Connor *et al.* (1997)]. Both probes were purified from agarose gels (QIAEX II Gel Extraction Kit, Qiagen, Valencia, CA). The GAPDH probe was generated from pGEM-3zf(+) containing a 780-bp human GAPDH cDNA (kindly provided by Dr. X. Y. Fu, Yale University). Probe radiolabeling was performed using the Random Primed DNA Labeling Kit (Boehringer Mannheim) and [α-^{32}P]dCTP (3000 Ci/mmol, NEN), followed by Sephadex G-50 spin-column chromatography (QuickSpin Columns, Boehringer Mannheim). Quantitative analysis was performed using a computing densitometer (Molecular Dynamics) and ImageQuant software.

RESULTS AND DISCUSSION

To identify target genes regulated by β_1 integrin variants, we have generated stable cell transfectants expressing human β_{1A} or β_{1C} under the control of a doxycycline-inducible promoter, using β_1-deficient GD25 cells (Fig. 1). Surface expression of β_{1A} and β_{1C} was comparable in GD25 stable cell transfectants, as shown in Fig. 1, using representative clones. Both β_{1C} and β_{1A} appeared to form surface-expressed heterodimers as evidentiated by coimmunoprecipitation of the endogenous α_6 subunit with either β_{1C} or β_{1A} (Fig. 2). Three clones were selected for each cell type and analysis of their adhesion properties showed that β_{1C} and β_{1A} transfectants adhered in a comparable manner to fibronectin and vitronectin substrates (Fig. 3 and not shown). β_1-deficient GD25 parental cells adhered to both substrates in an α_v-mediated manner as shown by an inhibitory antibody to α_v. In these cells, exogeneus β_{1C} or β_{1A} had a predominant role in mediating attachment to fibronectin (Fig. 3). Adhesion to and migration on fibronectin of β_{1C}- and β_{1A}-expressing cells occurred in a comparable manner both in GD25 and in CHO stable cell transfectants [Fornaro *et al.* (2000) and not shown].

Screening of 1176 murine cDNAs using first-strand cDNA of mRNA isolated from either β_{1C}- or β_{1A}-expressing cells that were attached to fibronectin showed a striking differential expression of few genes. The differential expression of two genes, MCP-3 and BRCA2, was reproduced in two independent cDNA array screening assays (Fig. 4 and not shown) and confirmed by Northern blot analysis (Figs. 5 and 6). A selective increase in MCP-3 and a decrease in BRCA2 mRNA levels in cells expressing β_{1C} compared to those in cells expressing β_{1A} were observed.

In conclusion, β_{1C} and β_{1A} variants differentially regulate mRNA expression levels of MCP-3 and BRCA2, whose products mediate, respectively, chemotaxis *in vitro* and *in vivo* (Haelens *et al.*, 1996; Fioretti *et al.*, 1998) and DNA repair as well as embryonic development (Welcsh *et al.*,

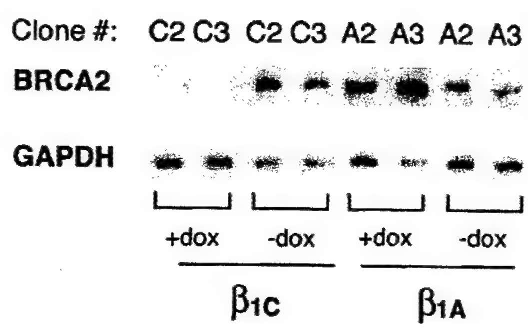

FIG. 6. Decreased BRCA2 mRNA levels in β_{1C}-GD25 cells. Total RNA (10 μg) was isolated from β_{1C}-GD25 cells (clones: C2, C3) and β_{1A}-GD25 cells (clones: A2, A3) grown as described in the legend to Fig. 4, either in the presence or in the absence of 2 μg/ml doxycycline. Total RNA was fractionated by agarose–formaldheyde gel electrophoresis, transferred to Hybond N+ membranes, and hybridized using a 5.7-kb BRCA2 cDNA probe. GAPDH cDNA was used as a probe to control for RNA loading.

2000). Since β_{1C}- and β_{1A}-expressing cells showed comparable adhesion to fibronectin, upregulation of MCP-3 and downregulation of BRCA2 mRNA levels in β_{1C}-expressing cells did not appear to be due to a differential ability of β_{1C} cells to adhere to the β_1 ligand fibronectin. Several cell functions such as cell cycle progression and proliferation as well as cyclin A gene regulation are controlled by cell adhesion to the ECM and spreading via integrins (Bottazzi and Assoian, 1997); similarly, loss of cell anchorage to the ECM has been shown to upregulate the expression of the cyclin-dependent kinase inhibitors $p27^{kip1}$ and $p21^{cip1/waf1}$ while, at the same time, decreasing the levels of cyclin A (Bottazzi and Assoian, 1997). However, changes in expression of cell cycle molecules have been shown to occur in response to β_{1C} integrin expression in an adhesion-independent manner (Fornaro *et al.*, 1999). The data presented in this study provide additional evidence that β_1 integrin cytoplasmic domain variants control expression of downstream target genes in a differential manner without affecting cell adhesion.

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Differential Role of β_{1C} and β_{1A} Integrin Cytoplasmic Variants in Modulating Focal Adhesion Kinase, Protein Kinase B/AKT, and Ras/Mitogen-activated Protein Kinase Pathways

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The integrin cytoplasmic domain modulates cell proliferation, adhesion, migration, and intracellular signaling. The β_1 integrin subunits, β_{1C} and β_{1A}, that contain variant cytoplasmic domains differentially affect cell proliferation; β_{1C} inhibits proliferation, whereas β_{1A} promotes it. We investigated the ability of β_{1C} and β_{1A} to modulate integrin-mediated signaling events that affect cell proliferation and survival in Chinese hamster ovary stable cell lines expressing either human β_{1C} or human β_{1A}. The different cytodomains of either β_{1C} or β_{1A} did not affect either association with the endogenous α_2, α_V, and α_5 subunits or cell adhesion to fibronectin or TS2/16, a mAb to human β_1. Upon engagement of endogenous and exogenous integrins by fibronectin, cells expressing β_{1C} showed significantly inhibited extracellular signal–regulated kinase (ERK) 2 activation compared with β_{1A} stable cell lines. In contrast, focal adhesion kinase phosphorylation and Protein Kinase B/AKT activity were not affected. Selective engagement of the exogenously expressed β_{1C} by TS2/16 led to stimulation of Protein Kinase B/AKT phosphorylation but not of ERK2 activation; in contrast, β_{1A} engagement induced activation of both proteins. We show that Ras activation was strongly reduced in β_{1C} stable cell lines in response to fibronectin adhesion and that expression of constitutively active Ras, Ras 61 (L), rescued β_{1C}-mediated down-regulation of ERK2 activation. Inhibition of cell proliferation in β_{1C} stable cell lines was attributable to an inhibitory effect of β_{1C} on the Ras/MAP kinase pathway because expression of activated MAPK kinase rescued β_{1C} antiproliferative effect. These findings show that the β_{1C} variant, by means of a unique signaling mechanism, selectively inhibits the MAP kinase pathway by preventing Ras activation without affecting either survival signals stimulated by integrins or cellular interactions with the extracellular matrix. These findings highlight a role for β_1-specific cytodomain sequences in maintaining an intracellular balance of proliferation and survival signals.

INTRODUCTION

Integrins are a large family of heterodimeric transmembrane receptors composed of α and β subunits (Hynes, 1992). In addition to their role as adhesion receptors, integrins have been shown to regulate intracellular signaling pathways and cellular functions such as cell migration, proliferation, and survival (Schwartz *et al.*, 1995; Bottazzi and Assoian, 1997; Frisch and Ruoslahti, 1997).

It is well established that the cytoplasmic domain of the β subunit is required for integrins to modulate many cellular functions and to trigger signaling events that result in protein phosphorylation (Hemler *et al.*, 1995; Fornaro and Languino, 1997; Wei *et al.*, 1998) and interactions with intracellular proteins (Hemler, 1998). Thus, mutations or deletions in the β_{1A} subunit cytodomain have been shown to alter the ability of this integrin to trigger focal adhesion kinase (FAK) phosphorylation (Guan *et al.*, 1991) and to interact with cytoskeletal proteins such as talin and filamin (Chen *et al.*, 1995; Lewis and Schwartz, 1995; Pfaff *et al.*, 1998).

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Abbreviations used: AKT, Protein Kinase B/AKT; CHO, Chinese hamster ovary; ERK, extracellular signal–regulated kinase; FAK, focal adhesion kinase; ILK, integrin-linked kinase; MEK, MAPK kinase; MEK EE, constitutively active MEK; MEK WT, wild-type MEK; PI 3-kinase, phosphatidylinositol 3-kinase.

The identification and characterization of a number of spliced variants of the integrin cytoplasmic domain in the β and α subgroups (Fornaro and Languino, 1997) have added a new level of complexity to integrin functions. Four different β_1 isoforms have been identified (β_{1A}, β_{1B}, β_{1C}, and β_{1D}) and have been shown to differentially affect receptor localization, cell proliferation, cell adhesion and migration, interactions with intracellular proteins, and ultimately phosphorylation and activation of signaling molecules (Belkin *et al.*, 1997; Fornaro and Languino, 1997; Belkin and Retta, 1998; Pfaff *et al.*, 1998; Retta *et al.*, 1998; Meredith *et al.*, 1999).

The β_{1C} integrin is an alternatively spliced variant of the β_1 subfamily that contains a unique 48-amino acid sequence in its cytoplasmic domain (Languino and Ruoslahti, 1992). We and others have shown that either full-length β_{1C} or its cytoplasmic domain inhibits prostate cancer epithelial cell (Fornaro *et al.*, 1998; Meredith *et al.*, 1999), endothelial cell (Meredith *et al.*, 1999), and fibroblast (Fornaro *et al.*, 1995; Meredith *et al.*, 1995, 1999) proliferation. In vivo, β_{1C} is expressed in nonproliferative, differentiated epithelium and is selectively down-regulated in prostatic adenocarcinoma, and its expression inversely correlates with markers of cell proliferation in breast carcinoma (Fornaro *et al.*, 1996, 1998, 1999; Manzotti *et al.*, 2000). However, the signaling pathways affected by β_{1C} are still unknown.

FAK is a nonreceptor protein tyrosine kinase that has been shown to colocalize with integrins at focal contact sites (Guan *et al.*, 1991). FAK becomes tyrosine phosphorylated in response to integrin engagement (Guan *et al.*, 1991; Kornberg *et al.*, 1991) and has been shown to prevent apoptosis (Frisch *et al.*, 1996; Hungerford *et al.*, 1996; Xu *et al.*, 1996; Illic *et al.*, 1998; Cary and Guan, 1999). Two recent reports have highlighted a new role for FAK in the modulation of cell cycle progression and in the inhibition of integrin-stimulated signaling events during mitosis (Zhao *et al.*, 1998; Yamakita *et al.*, 1999). The first study showed that FAK overexpression accelerates the G1/S phase transition, increases cyclin D1 levels, and decreases $p21^{waf1}$ expression (Zhao *et al.*, 1998). The second study demonstrated that FAK undergoes mitosis-specific serine phosphorylation accompanied by tyrosine dephosphorylation, which results in FAK/Cas/c-Src complex dissociation and inhibition of signal transduction pathways involving integrins (Yamakita *et al.*, 1999).

In addition to stimulating FAK, integrins can also activate the phosphatidylinositol 3-kinase (PI 3-kinase) pathway (Keely *et al.*, 1998). PI 3-kinases are a family of lipid kinases activated by a wide variety of extracellular stimuli. The lipid products of PI 3-kinases, specifically phosphatidylinositol (3,4)biphosphate and phosphatidylinositol(3,4,5)triphosphate, affect cell proliferation, survival, differentiation, and migration by targeting specific signaling molecules such as the serine/threonine protein kinase B, also known as AKT, and PKC (Jiang *et al.*, 1999; Rameh and Cantley, 1999). Integrin-mediated adhesion to the extracellular matrix stimulates the production of phosphatidylinositol(3,4)biphosphate and phosphatidylinositol(3,4,5)triphosphate (Khwaja *et al.*, 1997; King *et al.*, 1997), the association of the p85 PI 3-kinase subunit with FAK (Chen and Guan, 1994), and AKT activation (Khwaja *et al.*, 1997; King *et al.*, 1997). AKT plays an important role in transducing survival signals in response to several growth factors and integrin engagement (Khwaja *et al.*, 1997; Downward, 1998).

The small GTPase Ras is a critical component of signaling pathways that control cell proliferation, differentiation, and survival (Campbell *et al.*, 1998; Rebollo and Martinez-A, 1999). The Ras/extracellular signal–regulated kinase (ERK) 1 and 2/MAP kinase pathway plays a pivotal role in modulating gene expression and cell cycle progression in response to mitogens (Robinson and Cobb, 1997; Guadagno and Ferrell, 1998; Brunet *et al.*, 1999). Integrin clustering has been shown to stimulate Ras GTP loading (Clark and Hynes, 1996; Wary *et al.*, 1996; King *et al.*, 1997; Mainiero *et al.*, 1997; Miranti *et al.*, 1999) and to activate specific effectors of the Ras/MAP kinase signaling cascade such as Raf-1 and MAPK kinase (MEK) (Howe *et al.*, 1998; Schlaepfer and Hunter, 1998). In several studies, the dominant negative N17 mutant of Ras has been shown to block extracellular matrix–mediated ERK2 activation (Clark and Hynes, 1996; Wary *et al.*, 1996; King *et al.*, 1997; Mainiero *et al.*, 1997; Schlaepfer and Hunter, 1997; Wei *et al.*, 1998), whereas in one report it had no effect (Chen *et al.*, 1996b). The mechanisms of integrin-mediated activation of the MAP kinase cascade comprise three models (Howe *et al.*, 1998). Two models include Src family kinases and Ras as critical links between integrin-mediated adhesion and MAP kinase activation. In the first model, integrin ligation leads to Src and FAK activation, Grb2 binding to FAK, and membrane localization of the guanine nucleotide exchange factor Sos, which then promotes Ras activation (Schlaepfer *et al.*, 1994, 1998). In the second model, integrins activate the Ras/MAP kinase pathway via the tyrosine kinase Fyn and the adaptor protein Shc (Wary *et al.*, 1996, 1998). A recent report has indicated that fibronectin-induced PKC activation plays a role in ERK2 activation upstream of Shc (Miranti *et al.*, 1999). The third model proposes a Ras-independent activation of Raf and, thus, ERK2 by integrins (Chen *et al.*, 1996b; Lin *et al.*, 1997).

Using Chinese hamster ovary (CHO) stable cell lines expressing either human β_{1C} or human β_{1A}, we have analyzed the ability of β_{1C} and β_{1A} to modulate signaling pathways that control cell proliferation and survival. The β_{1C} variant associates with the same α subunits as β_{1A} and does not affect cell adhesion to β_1 ligands. We show that β_{1C} has an inhibitory effect on ERK2 activation mediated by fibronectin without affecting FAK phosphorylation and AKT activity. We also show that Ras activation stimulated by adhesion to fibronectin is inhibited in β_{1C} transfectants and that constitutively active Ras and MEK rescue β_{1C}-mediated down-regulation of ERK2 activation and inhibition of cell growth, respectively. This is the first description of a selective inhibitory role of the integrin cytoplasmic domain on the Ras/MAP kinase pathway. Moreover, AKT phosphorylation is observed in response to antibody-mediated engagement of human β_{1C} and β_{1A}, and ERK2 activation is supported by β_{1A} but not by β_{1C} ligation, indicating a different role for β_1 variants in the activation of AKT and MAP kinase pathways. We suggest that by expressing variant β_1 intracellular domains, cells may accomplish the delicate task of inhibiting proliferation without affecting either selective downstream sur-

vival signals (FAK and AKT) mediated by integrins or interactions with the extracellular environment.

MATERIALS AND METHODS

Reagents and Antibodies

Rabbit antibodies specific for the β_{1C} subunit cytoplasmic domain were affinity-purified as described previously (Fornaro *et al.*, 1996). The following antibodies were used: mouse mAbs P4C10 and TS2/16 to human β_1 integrin (Life Technologies, Gaithersburg, MD, and American Type Culture Collection, Rockville, MD, respectively), 7E2 to hamster β_1 integrin, PB1 to hamster $\alpha_5\beta_1$ (a kind gift of Dr. R.L. Juliano, University of North Carolina, Chapel Hill, NC), E10 to phospho-ERK1 and 2 (New England Biolabs, Beverly, MA), 12CA5 to hemagglutinin (Boehringer Mannheim, Indianapolis, IN), and to pan Ras (Transduction Laboratories, Lexington, KY); rabbit affinity-purified antibodies to FAK Y^{397} (Biosource International, Camarillo, CA), to AKT (New England Biolabs), and to FAK and ERK1 and 2 (Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit antisera to α_5, α_v, or α_4 were provided by Dr. E. Ruoslahti (The Burnham Institute, La Jolla, CA), antiserum to α_2 was provided by Dr. M.E. Hemler (Dana-Farber Cancer Institute, Boston, MA), and antiserum to β_{1C} was described previously (Fornaro *et al.*, 1996). Human plasma fibronectin and human vitronectin were purified as described (Engvall and Ruoslahti, 1977; Yatohgo *et al.*, 1988). Poly-L-lysine and nonimmune rabbit and mouse immunoglobulin G were purchased from Sigma Chemical (St. Louis, MO).

Cells and Plasmids

To obtain stable cell lines expressing β_{1A} in a tetracycline-regulated system, *ClaI–XbaI* fragment encoding full-length human β_{1A} was isolated from Bluescript-β_{1A} and subcloned into *ClaI–SpeI* sites in the pTet-Splice plasmid (a kind gift of Dr. D. Schatz, Yale University, New Haven, CT) to generate the pTet-β_{1A} construct. The pTet-β_{1C} construct has been described previously (Fornaro *et al.*, 1999). CHO stable cell lines expressing either human β_{1C} (clones 16.4, 16.28, and 16.30) or human β_{1A} (clones 10.2, 10.18, and 10.23) integrins under the control of a tetracycline-regulated promoter were generated and maintained in growth medium containing 1 μg/ml tetracycline (Boehringer Mannheim) and 0.1 mg/ml G418 (Life Technologies) as described (Fornaro *et al.*, 1999).

pMLC-1 plasmids containing hemagglutinin-tagged wild-type MEK (MEK WT) and constitutively active MEK (MEK EE) have been described previously (Bennett and Tonks, 1997). The pGEX-RBD plasmid encodes amino acids 1–149 of cRaf-1 fused to GST (Taylor and Shalloway, 1996). The pMT3-Ras 61 (L) encodes a c-ras^H form containing a codon 61 mutation (Bennett *et al.*, 1996).

β_{1C}-CHO stable cell lines were transiently transfected by electroporation by using 10 μg of either MEK WT, MEK EE, Ras 61 (L), or vector alone as described (Fornaro *et al.*, 1999). Cells were incubated for 48 h at 37°C in growth medium either in the absence or in the presence of 1 μg/ml tetracycline and serum-starved during the last 24 h of the 48-h culture before analysis of either cell proliferation or ERK2 activity as described below.

Flow Cytometry

Surface expression of exogenous human β_{1C} and β_{1A} integrins was achieved by withdrawal of tetracycline from the growth medium; in both cell transfectants, maximal and comparable β_{1C} or β_{1A} expression were consistently obtained 48 h after tetracycline removal. For each experiment, exogenous human β_1 integrin expression was monitored by FACS with TS2/16 serum-free culture supernatant or 12CA5 as negative control antibody (Fornaro *et al.*, 1999). Endogenous hamster β_1 or $\alpha_5\beta_1$ integrin expression was analyzed with either 5 μg/ml 7E2 or 1 μg/ml PB1, respectively (Fornaro *et al.*, 1995).

Immunoprecipitation of β_{1C} and β_{1A} Integrins

CHO stable cell lines were cultured for 48 h in the absence of tetracycline to induce β_{1C} or β_{1A} integrin expression (Fornaro *et al.*, 1999). Cells were detached with 0.05% trypsin/0.53 mM EDTA (Life Technologies) and surface iodinated as described previously (Bartfeld *et al.*, 1993). Cells were lysed in 1% NP-40 (Calbiochem, La Jolla, CA), 0.5% sodium deoxycholate (Sigma), 0.1% SDS (American Bioanalytical, Natick, MA), 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM PMSF (Life Technologies), 10 μg/ml aprotinin (Sigma), 10 μg/ml leupeptin (Calbiochem), and 10 μg/ml pepstatin (Sigma) for 30 min at 4°C. β_{1C} and β_{1A} integrins were immunoprecipitated with P4C10 and protein A–Sepharose (Sigma) as described (Fornaro *et al.*, 1995). Immunocomplexes were dissociated with 20 mM Tris-HCl, pH 7.5, 2% SDS and boiled for 5 min. The dissociated material was then diluted 10-fold with lysis buffer and reprecipitated overnight at 4°C with 30 μl of rabbit serum specific for the β_{1C} subunit cytoplasmic domain. Immunoprecipitates were recovered with protein A–Sepharose, washed four times with lysis buffer, and resuspended in loading buffer (2% SDS, 50 mM Tris-HCl, pH 6.8, 100 mM DTT [American Bioanalytical], 10% glycerol, and 0.1% bromphenol blue [Bio-Rad, Hercules, CA]). Proteins were separated by SDS-PAGE (7.5%) and visualized by autoradiography.

Immunoprecipitations of α Subunits Associated with β_{1C}

CHO stable cell lines were cultured for 72 h in the absence of tetracycline; cells were then detached with 0.05% trypsin/0.53 mM EDTA and surface iodinated as described above. Cells were lysed in 1% Triton X-100 (American Bioanalytical), 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 30 min at 4°C. β_{1C} and β_{1A} integrins were immunoprecipitated with P4C10 as described above. Immunoprecipitates were washed five times with lysis buffer, resuspended in 10 mM Tris-HCl, pH 7.5, 0.5% SDS, and incubated for 10 min at 70°C. The eluted material was diluted threefold with lysis buffer and reprecipitated with rabbit antiserum to α_5, α_V, α_4, or α_2 overnight at 4°C. Immunoprecipitates were recovered with protein A–Sepharose, washed three times with lysis buffer, and resuspended in loading buffer. Proteins were separated by SDS-PAGE (10%) and visualized by autoradiography.

Cell Adhesion Assay to β_1 Ligands

Cell adhesion to fibronectin (10 μg/ml), 7E2 (1 μg/ml), TS2/16 (1:10 dilution of culture supernatant), mouse immunoglobulin G (1 μg/ml), and BSA (10 mg/ml; Sigma) was performed as described previously (Languino *et al.*, 1993) with 25,000 ^{51}Cr-labeled cells (^{51}Cr from DuPont–New England Nuclear, Wilmington, DE).

For analysis of FAK, AKT, and ERK2, CHO stable cell lines were cultured for 48 h either in the absence or in the presence of 1 μg/ml tetracycline, starved, and then detached as described above. Cells were held in suspension for 30–60 min at 37°C and either kept in suspension or plated on tissue culture plates coated with poly-L-lysine (5–10 μg/ml), fibronectin (10 μg/ml), TS2/16 (1:10 dilution of culture supernatant), or 7E2 (3 μg/ml) at 37°C for the indicated times. Where indicated, cells were incubated for 15 min at 37°C with 100 nM wortmannin (Calbiochem) before plating onto ligand-coated dishes. The cells were then washed twice with PBS (Life Technologies) and lysed in the appropriate ice-cold lysis buffer. The protein content in each lysate was quantitated with the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL).

In all instances, quantification of immunoreactive bands was performed by densitometric analysis; the values are given as fold increase on fibronectin, TS2/16, or 7E2 versus poly-L-lysine or suspension within each established cell line after normalization for protein loading. The values from several experiments are reported as means ± SEM.

FAK Analysis

CHO stable cell lines were lysed with 1% NP-40, 0.5% deoxycholate, 50 mM HEPES, pH 7.5, 150 mM NaCl, 100 mM sodium fluoride (Sigma), 1 mM sodium vanadate (Sigma), 5 mM $Na_4P_2O_7$ (J.T. Baker, Phillipsburg, NJ), 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 30 min at 4°C, and insoluble material was removed by centrifugation at 14,000 × *g* for 15 min at 4°C.

FAK was immunoprecipitated from 500 μg of total cell lysate with 0.5 μg of C-20, an affinity-purified antibody to FAK. Immunocomplexes were collected with protein A–Sepharose, washed five times with lysis buffer, and resuspended in loading buffer. Proteins were separated by 10% SDS-PAGE, and FAK phosphorylation on Tyr^{397} was analyzed by immunoblotting with a rabbit affinity-purified antibody that recognizes FAK only when phosphorylated on Y^{397}. FAK protein levels were analyzed by immunoblotting with C-20 rabbit affinity-purified antibody to FAK as described (Zheng *et al.*, 1999).

AKT Analysis

CHO stable cell lines were lysed with 1% NP-40, 50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM EDTA, 50 mM sodium fluoride, 1 mM sodium vanadate, 1 mM $Na_4P_2O_7$, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 30 min at 4°C. Analysis of AKT phosphorylation was performed by immunoblotting with phospho-specific antibody to Ser^{473} (New England Biolabs) according to the manufacturer's instructions.

AKT kinase activity was assayed according to Franke *et al.* (1995). Briefly, 50 μg of detergent cell extracts were cleared by centrifugation at 14,000 × *g* for 15 min at 4°C. AKT was immunoprecipitated with 0.1 μg of affinity-purified antibody to AKT. Immunocomplexes were collected with protein A–Sepharose and washed three times with lysis buffer, once with 20 mM HEPES, pH 7.5, and once with kinase buffer (20 mM HEPES, pH 7.5, 1 mM DTT, 10 mM $MnCl_2$, 10 mM $MgCl_2$). The AKT kinase activity was assayed with kinase buffer containing 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Life Sciences, Arlington Heights, IL), 5 μM ATP (Boehringer Mannheim), and 100 μg/ml histone H2B (Boehringer Mannheim) as a substrate for 20 min at 30°C. The reactions were terminated with loading buffer. Phosphorylated histone H2B was viewed by autoradiography.

ERK2 Analysis

CHO stable cell lines were lysed as described for analysis of AKT activation. Analysis of ERK2 phosphorylation by immunoblotting was performed with 0.5 μg/ml E10, a mAb that recognizes ERK2 only when phosphorylated at Thr^{202}/Tyr^{204}, according to the manufacturer's instructions (New England Biolabs). ERK2 activation was analyzed by in vitro kinase assay with myelin basic protein as described (Fornaro *et al.*, 1999).

Assay for Detection of Activated Ras

Ras activation was analyzed as described previously (Taylor and Shalloway, 1996). Briefly, GST-RBD expression in transformed *Escherichia coli* DH5α was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (American Bioanalytical) for 2 h at 37°C. The cells were then washed once with ice-cold 20 mM HEPES, pH 7.5, 150 mM NaCl and lysed by sonication in the following buffer: 20 mM HEPES, pH 7.5, 120 mM NaCl, 10% glycerol, 2 mM EDTA, 100 mg/ml lysozyme (American Bioanalytical), 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin. The lysate was clarified by centrifugation and incubated with glutathione Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) for 30 min at 4°C. The Sepharose beads were then washed six times with lysis buffer containing 0.5% NP-40 and stored in the same buffer at 4°C.

For affinity precipitation, cells were washed twice with ice-cold 20 mM HEPES, pH 7.5, 150 mM NaCl and lysed with the following buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% deoxycholate, 10% glycerol, 10 mM $MgCl_2$, 25 mM sodium fluoride, 1 mM EDTA, 1 mM sodium vanadate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for 30 min at 4°C. One milligram of whole cell lysate was incubated with GST-RBD bound to glutathione Sepharose for 30 min at 4°C. Bound proteins were washed three times with lysis buffer, eluted with loading buffer, and separated by SDS-PAGE (12%). Proteins were visualized by immunoblotting with 2 μg/ml anti-pan Ras mouse mAb according to the manufacturer's instructions (Transduction Laboratories).

Proliferation Assay

CHO stable cell lines were cultured for 48 h either in the absence or in the presence of 1 μg/ml tetracycline, starved during the last 24 h of the 48-h culture, and then detached with 0.05% trypsin/0.53 mM EDTA. Cells were resuspended in serum-free medium and plated (2,500–20,000 cells/well) on either 96- or 24-well plates coated with 1 μg/ml fibronectin for 1 h at 37°C. Attached cells were cultured for 72–96 h at 37°C in growth medium containing 5% FCS either in the absence or in the presence of 1 μg/ml tetracycline. Cells were washed, fixed with 3% paraformaldehyde, and stained overnight with 0.5% toluidine blue. Triplicate observations were performed. Two to 10 fields/well were randomly chosen and counted by microscopic examination. The results are expressed as number of cells per well. Group differences were compared with one-way analysis of variance.

RESULTS

Analysis of α Subunits Associated with β_{1C} and of β_{1C}-CHO Cell Adhesion

CHO stable cell lines expressing either human β_{1C} or human β_{1A} under the control of a tetracycline-regulated promoter were characterized for their ability to associate with α subunits and to adhere to integrin ligands. Exogenous expression of either β_{1C} or β_{1A} in CHO cells was analyzed by FACS with TS2/16 mAb to human β_1 integrin; comparable levels of surface expression of β_{1C} and β_{1A} were consistently obtained in all the experiments 48 h after tetracycline removal (Figure 1, A and B). In parallel, the levels of endogenous β_1 were evaluated in both β_{1C} and β_{1A} CHO stable cell lines by FACS with 7E2 mAb to hamster β_1 integrin (Figure 1, A and B). Exogenous expression of either β_{1C} or β_{1A} was completely prevented by tetracycline (Figure 1, C and D). The expression of human β_{1C} and β_{1A} was also analyzed by immunoprecipitation from detergent cell extracts of ^{125}I-labeled CHO cells. P4C10, a mAb to the human β_1 extracellular domain, immunoprecipitated surface-expressed integrin complexes containing either β_{1C} or β_{1A} (Figure 1E, lanes 2 and 4). P4C10 immunocomplexes were reprecipitated with rabbit serum to the β_{1C} cytodomain. These results confirm appropriate β_{1C} cell surface expression (Figure 1E, lane 6). To characterize the α subunits associated with β_{1C}, P4C10 immunocomplexes were reprecipitated with rabbit serum against α_2, α_V, or α_5, which are known to be associated predominantly with β_1 in CHO cells (Takada *et al.*, 1992; Balzac *et al.*, 1993). As shown in Figure 2, both exogenous β_{1C} and β_{1A} were associated with endogenous α_2, α_V, or α_5 integrin subunits in CHO stable cell lines. The β_{1C}- and β_{1A} CHO stable cell lines also attached in a comparable manner to increasing concentrations of fibronectin, 7E2, or TS2/16; no differences were observed in the number of attached cells at 30, 90, or 120 min (Figure 3; our unpublished results).

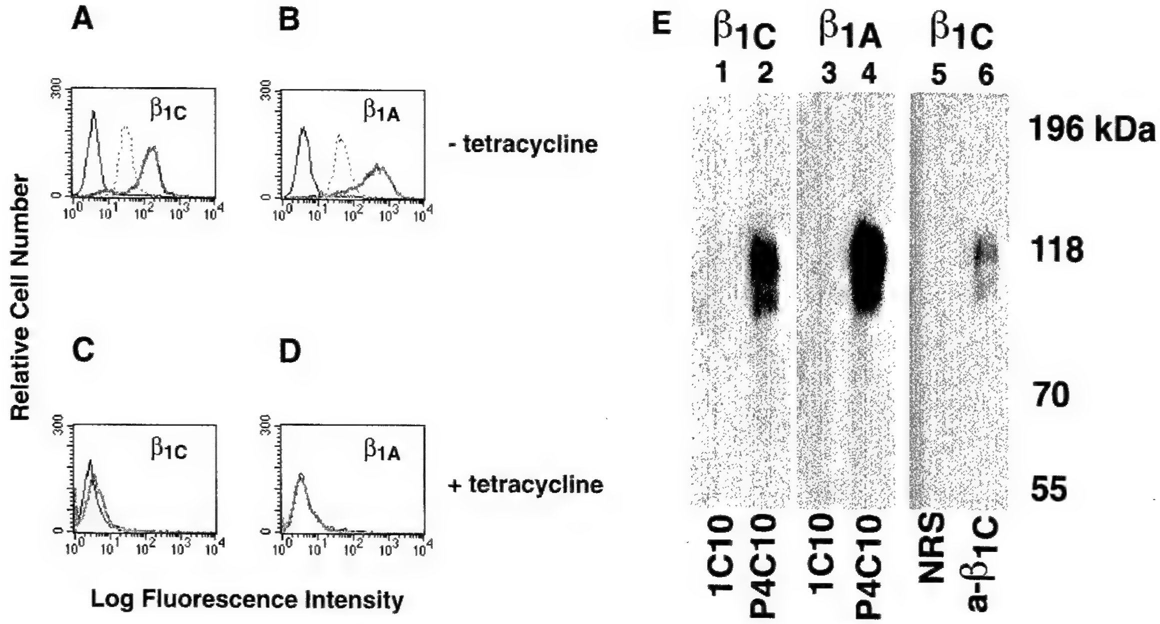


Figure 1. Surface expression of β_{1C} and β_{1A} in CHO cells. (A–D) β_{1C} or β_{1A} CHO stable cell lines were cultured for 48 h either in the absence (A and B) or in the presence (C and D) of 1 μg/ml tetracycline and analyzed by FACS with TS2/16 mAb to human β_1 integrin, 7E2 mAb to hamster β_1 integrin, or 12CA5 as a negative control, followed by FITC goat anti-mouse immunoglobulin G. Fluorescence intensity is expressed in arbitrary units. FACS analysis of a representative clone for each β_1 variant is shown. Thick gray line, TS2/16; dotted line, 7E2; thin black line, 12CA5. (E) CHO stable cell lines were cultured as in A and B and surface-labeled with Na ^{125}I; exogenous β_1 integrins were immunoprecipitated with P4C10 mAb to human β_1 integrin (lanes 2 and 4). The immunoprecipitated material was then eluted from protein A–Sepharose with 50 mM Tris-HCl, pH 7.5, 2% SDS and boiled for 5 min. The immunocomplexes were then reprecipitated with rabbit antiserum to the β_{1C} cytoplasmic domain (lane 6) and separated on 7.5% SDS-PAGE. mAb 1C10 (lanes 1 and 3) or normal rabbit serum (lane 5) were used as negative controls. Lanes 1, 2, 5, and 6, β_{1C} CHO; lanes 3 and 4, β_{1A} CHO. Proteins were visualized by autoradiography. Prestained marker proteins (in kilodaltons) are shown.

β_{1C} Integrin Expression Does Not Affect FAK Phosphorylation or AKT Activation

To analyze the effect of β_{1C} on integrin-mediated intracellular signaling pathways, we used the CHO stable cell lines described above (Figures 1 and 2). It has been shown that integrin ligation leads to tyrosine phosphorylation of intracellular proteins, including FAK (Schwartz *et al.*, 1995).

To examine whether FAK phosphorylation was differentially affected by β_{1C} and β_{1A} integrin variants, FAK was immunoprecipitated from detergent cell extracts prepared from either β_{1C} or β_{1A} stable cell lines. FAK phosphorylation was analyzed by immunoblotting with an antibody that recognizes FAK only when phosphorylated on Tyr^{397} (Sieg *et al.*, 1999). As shown in Figure 4, cell adhesion to fibronectin induced FAK phosphorylation on Tyr^{397} in both β_{1C} and β_{1A} stable cell lines compared with cells in suspension (top panel). The results indicate that β_{1C} integrin expression does not affect FAK phosphorylation mediated by adhesion to fibronectin.

We then examined the ability of β_{1C} and β_{1A} integrins to activate AKT, a downstream effector of PI 3-kinase that promotes cell survival. AKT activity was first assayed on detergent cell extracts obtained from cells that attached to fibronectin for 10 or 30 min. As shown in Figure 5A, adhesion to fibronectin for 10 min induced comparable activation of AKT in both β_{1C} and β_{1A} stable cell lines as determined by in vitro kinase assay (top panel, lanes 2 and 5). However, upon adhesion to fibronectin for 30 min, a modest but consistent increase of AKT activation was observed in β_{1C} versus β_{1A} stable cell lines (top panel, lanes 3 and 6). Similar results were obtained by immunoblotting with a phospho-specific AKT antibody (Figure 5B); total lysates from cells that were either held in suspension or allowed to adhere for 30 min to fibronectin, TS2/16, or 7E2 were immunoblotted with phospho-specific antibody to Ser^{473}. As shown in panel B, a marked increase in AKT serine phosphorylation was observed in β_{1C} and β_{1A} stable cell lines upon adhesion to fibronectin (top panel, lanes 2 and 6), 7E2 (top panel, lanes 3 and 7), or TS2/16 (top panel, lanes 4 and 8) compared with cells in suspension and with cells on poly-L-lysine (our unpublished results). These results indicate that both β_1 variants activate AKT in CHO cells. Densitometric analysis performed on three separate experiments showed that cell adhesion to fibronectin, TS2/16, or 7E2 induced an increase in AKT Ser^{473} phosphorylation in β_{1C} (6.3 $\pm$ 1.4-fold, 4.4 $\pm$ 0.8-fold, and 5.5 $\pm$ 1.9-fold increase, respectively) as well as in β_{1A} (2.2 $\pm$ 0.5-fold, 2.1 $\pm$ 0.2-fold, and 2.7 $\pm$ 0.7-fold increase, respectively) stable cell lines (our unpublished results). No differences in AKT activation were detected upon adhesion to fibronectin between β_{1C} and β_{1A} stable cell lines cultured in the presence of tetracycline (Figure 5C, lanes 2, 3, 5, and 6) to prevent expression of exogenous β_1 variants. AKT phosphorylation in response to engagement of either endogenous integrins or exogenous β_{1C} and β_{1A} variants by

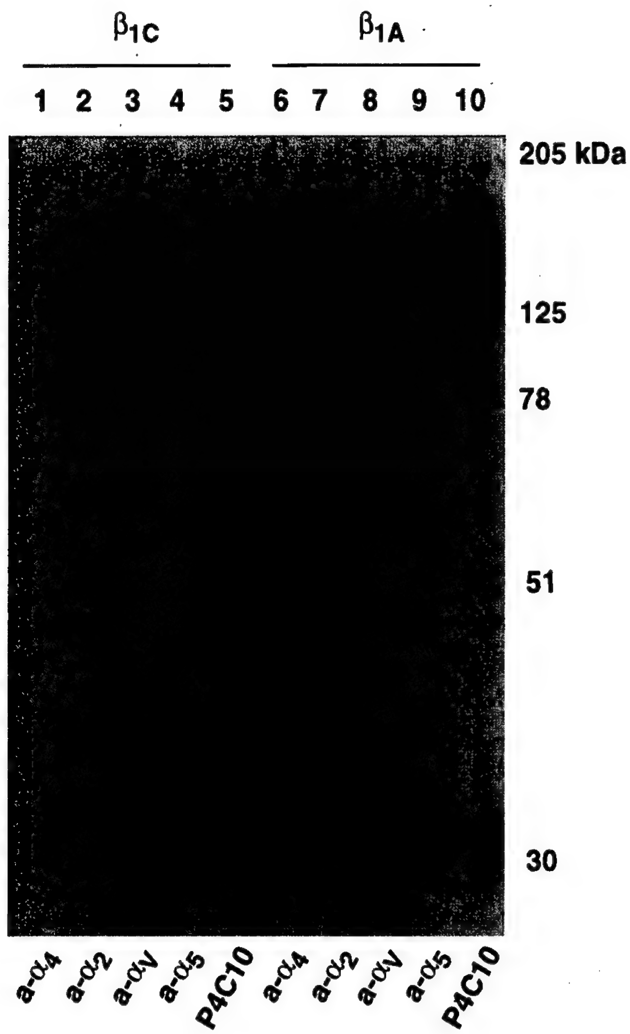


Figure 2. β_{1C} associates with α_5, α_V, and α_2 subunits. β_{1C} or β_{1A} CHO stable cell lines were cultured for 72 h in the absence of tetracycline and surface-labeled with iodine, and exogenous β_1 integrins were immunoprecipitated with P4C10 (lanes 5 and 10). The immunoprecipitated material was then eluted from protein A–Sepharose with 10 mM Tris-HCl, pH 7.5, 0.5% SDS for 10 min at 70°C, reprecipitated with rabbit antiserum to α_4 (lanes 1 and 6), α_2 (lanes 2 and 7), α_V (lanes 3 and 8), or α_5 (lanes 4 and 9), and separated by 10% SDS-PAGE. Lanes 1–5, β_{1C} CHO; lanes 6–10, β_{1A} CHO. Proteins were detected by autoradiography. Prestained marker proteins (in kilodaltons) are shown.

either fibronectin or TS2/16 was completely inhibited by wortmannin, a PI 3-kinase inhibitor (Figure 5B, top panel, lanes 10, 12, 14, and 16). These data show that β_{1C} and β_{1A} do not differentially affect PI 3-kinase/AKT pathway activation induced by fibronectin and that antibody-mediated engagement of β_{1C} and β_{1A} stimulates AKT phosphorylation.

β_{1C} Integrin Expression Inhibits MAP Kinase Activation Stimulated by Fibronectin

MAP kinase pathway activation by integrins is transient and is detectable soon after integrin engagement (maximum at

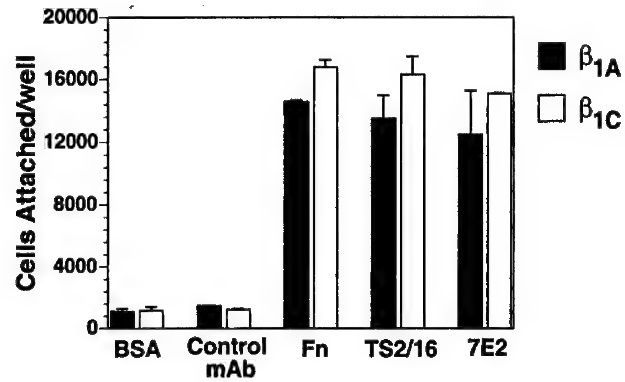


Figure 3. CHO cell adhesion is not affected by β_{1C} expression. β_{1C} or β_{1A} CHO stable cell lines were cultured as described for Figure 1. Cells were detached and labeled with ^{51}Cr in DMEM containing 10% FCS for 1 h at 37°C. Cells were then washed in serum-free medium, and 2.5×10^4 cells were allowed to adhere to fibronectin-coated (10 μg/ml), 7E2-coated (1 μg/ml), TS2/16-coated (1:10 dilution of culture supernatant), or negative control mAb-coated (1 μg/ml) or BSA-coated (10 mg/ml) wells at 37°C for 30 min. Attached cells were then washed and lysed, and radioactivity was measured by liquid scintillation counting. Duplicate observations with two separate clones for each β_1 variant were performed in each experiment, and the experiments were repeated at least twice with similar results.

10 min in CHO cells; Figure 6A). We examined the ability of β_{1C} and β_{1A} to modulate ERK2 activation in CHO stable cell lines. Endogenous and exogenous integrins were engaged with fibronectin (Figure 6, A–C), whereas exogenous human

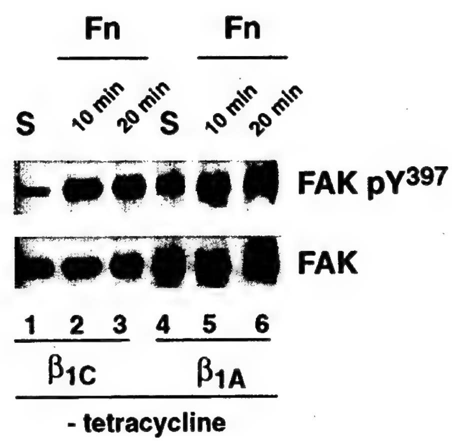


Figure 4. Expression of β_{1C} integrin does not affect FAK activation. β_{1C} or β_{1A} CHO stable cell lines were cultured for 48 h in the absence of tetracycline and serum-starved during the last 24 h of the 48-h culture. The cells were detached and either held in suspension (S; lanes 1 and 4) or plated on tissue culture plates coated with fibronectin (Fn; lanes 2, 3, 5, and 6) for either 10 or 20 min at 37°C. FAK was immunoprecipitated from 500 μg of total cell lysate with 0.5 μg of affinity-purified antibody to FAK, and its phosphorylation was analyzed by immunoblotting with 0.2 μg/ml phospho-specific antibody to Tyr[397]. FAK protein levels were analyzed with 0.1 μg/ml affinity-purified antibody to FAK, and proteins were visualized by enhanced chemiluminescence. The experiments were repeated twice with consistent results.

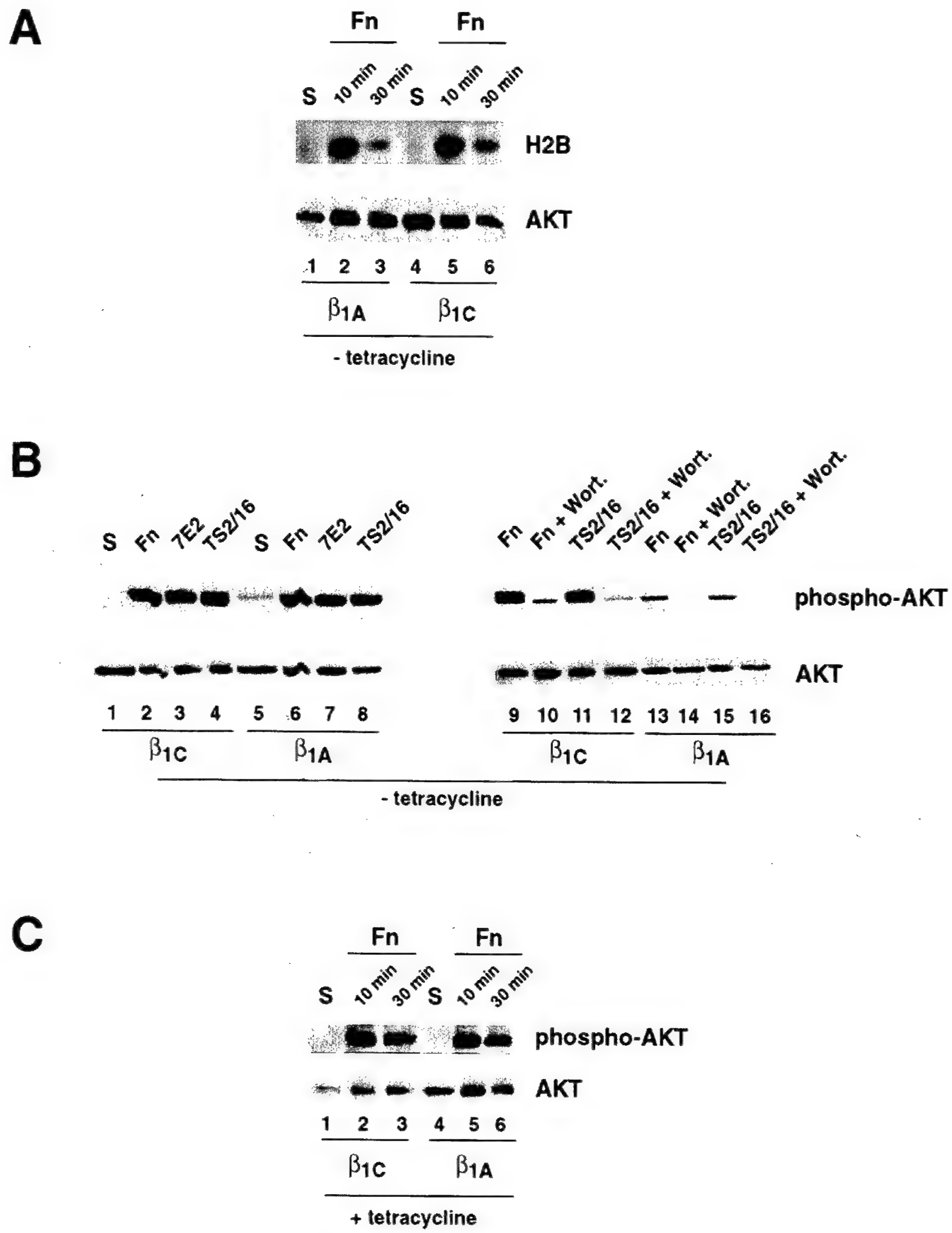


Figure 5. AKT activation in β_{1C} and β_{1A} transfectants. AKT activation was analyzed by in vitro kinase assay (A) and by immunoblotting (B and C). β_{1C} or β_{1A} CHO stable cell lines were cultured for 48 h either in the absence (A and B) or in the presence (C) of 1 μg/ml tetracycline and serum-starved during the last 24 h of the 48-h culture. The cells were detached and either held in suspension (S; A and C, lanes 1 and 4; B, lanes 1 and 5) or seeded on tissue culture plates coated with fibronectin (Fn; A and C, lanes 2, 3, 5, and 6; B, lanes 2, 6, 9, 10, 13, and 14), TS2/16 (B, lanes 4, 8, 11, 12, 15, and 16), or 7E2 (B, lanes 3 and 7) for either 10 min (A and C) or 30 min (A–C) at 37°C. Cells were also incubated with 100 nM wortmannin (Wort.) for 15 min at 4°C before plating on either fibronectin (B, lanes 10 and 14) or on TS2/16 (B, lanes 12 and 16). (A) AKT was immunoprecipitated from total cell lysate with 0.1 μg of affinity-purified antibody to AKT, and its kinase activity was analyzed by in vitro kinase assay with histone H2B (H2B) as a substrate. Phosphorylated H2B was visualized by autoradiography (top panel). (B and C) Detergent cell extracts were analyzed with 0.05 μg/ml phospho-specific antibody that recognizes AKT only when phosphorylated at Ser[473] (top panels). The levels of AKT expression were examined with 0.1 μg/ml control AKT antibody (phosphorylation state independent; A–C, bottom panels). Proteins were visualized by enhanced chemiluminescence. The experiments were repeated twice with consistent results.

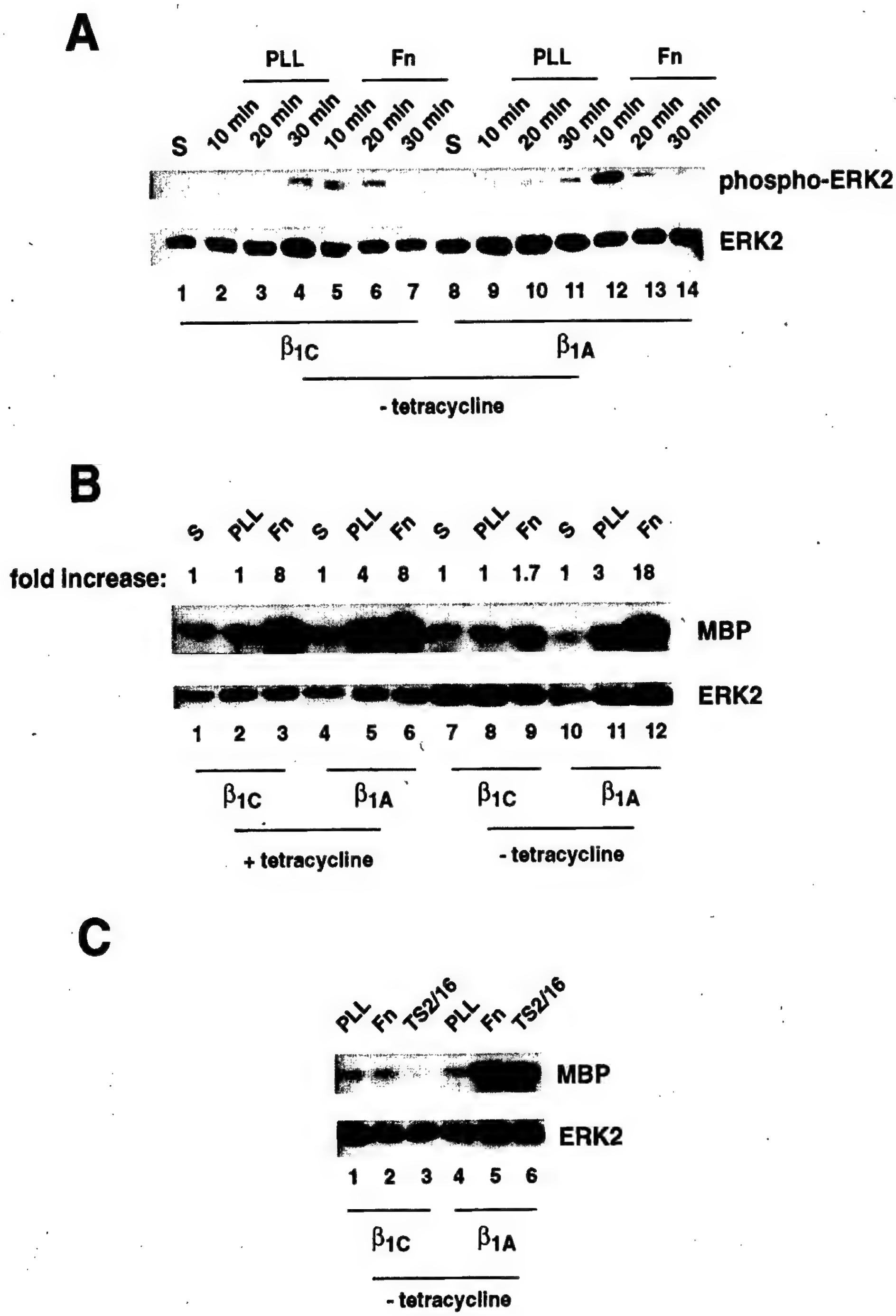


Figure 6. β_{1C} prevents ERK2 activation mediated by fibronectin. β_{1C} or β_{1A} CHO stable cell lines were cultured as described for Figure 5. The cells were detached and either held in suspension (S; A, lanes 1 and 8; B, lanes 1, 4, 7, and 10) or seeded on tissue culture plates coated with poly-L-lysine (PLL; A, lanes 2–4 and 9–11; B, lanes 2, 5, 8, and 11; C, lanes 1 and 4), fibronectin (Fn; A, lanes 5–7 and 12–14; B, lanes 3, 6, 9, and 12; C, lanes 2 and 5), or TS2/16 (C, lanes 3 and 6) for either 10 min (A–C) or 20 or 30 min (A) at 37°C. Cells were lysed, and ERK2 activation was analyzed by immunoblotting (A) or by in vitro kinase assay (B and C). (A) Detergent cell extracts were analyzed with 0.5 μg/ml mAb E10, which recognizes ERK2 only when phosphorylated at Thr^{202}/Tyr^{204} (top panel). (B and C) ERK2 was immunoprecipitated from 50 μg of total cell lysate with 0.5 μg of affinity-purified antibody to ERK2, and its kinase activity was analyzed by in vitro kinase assay with myelin basic protein (MBP) as a substrate. Phosphorylated MBP was visualized by autoradiography (top panels). The levels of expression of ERK2 were analyzed with 0.1 μg/ml rabbit affinity-purified antibody to ERK 2 (A–C, bottom panels). Proteins were visualized by enhanced chemiluminescence. In B, ERK2 activation is expressed as fold increase over the activity detected in cells held in suspension. The experiments were repeated at least twice with two separate clones for each variant with consistent results.

β_{1C} or exogenous human β_{1A} integrins were engaged with TS2/16 (Figure 6C). The activation of ERK2 was analyzed by immunoblotting with E10 mAb, which recognizes the Thr^{202}/Tyr^{204} phosphorylated form of ERK2 (Figure 6A, top panel), and by in vitro kinase assay (Figure 6, B and C, top panels); comparable amounts of ERK2 were used in the kinase assays (Figure 6, B and C, bottom panels). ERK2 activation was reduced significantly in β_{1C} compared with β_{1A} stable cell lines in response to integrin engagement by fibronectin as determined by immunoblotting (Figure 6A, top panel, lanes 5 and 12) and by in vitro kinase assay (Figure 6, B, top panel, lanes 9 and 12, and C, top panel, lanes 2 and 5). In the presence of tetracycline, adhesion to fibronectin mediated by endogenous integrins induced comparable ERK2 activation in both β_{1C} and β_{1A} stable cell lines (Figure 6B, top panel, lanes 3 and 6). Exogenous expression of β_1 variants in CHO cells did not alter the expression levels of endogenous hamster β_1 subunit or $\alpha_5\beta_1$ integrin as assessed by FACS analysis (Figure 1, A and B; our unpublished results), indicating that the differences in ERK2 activation on fibronectin between β_{1C}- and β_{1A}-expressing cells were not due to changes in endogenous $\alpha_5\beta_1$ integrin expression, the major fibronectin receptor in CHO cells.

Ligation of β_{1C} integrin by TS2/16 compared with poly-L-lysine did not induce activation of ERK2 as assessed by in vitro kinase assay (Figure 6C, top panel, lanes 1 and 3) or by immunoblotting with mAb E10 (our unpublished results). However, attachment of β_{1A} stable cell lines to TS2/16 resulted in activation of ERK2 compared with poly-L-lysine (Figure 6C, top panel, lanes 4 and 6). These results show that β_{1C} has an inhibitory effect on ERK2 activation mediated by fibronectin and, at variance with β_{1A}, is not able to stimulate ERK2 activity. These results also show that ERK2 activity is inhibited in cells attached to fibronectin for 10 min when both FAK and AKT are activated.

β_{1C} Integrin Expression Inhibits Fibronectin-mediated Ras Activation

Several reports have shown the role of Ras as an important effector of integrin-mediated activation of the MAP kinase pathway (Schlaepfer *et al.*, 1994, 1998; Clark and Hynes, 1996; Wary *et al.*, 1996; King *et al.*, 1997; Mainiero *et al.*, 1997; Schlaepfer and Hunter, 1997; Wei *et al.*, 1998). The data presented above indicate that β_{1C} has an inhibitory effect on ERK2 activity. Therefore, to determine whether β_{1C} mediated this effect at the level of Ras, Ras activation was assessed through its ability to bind the Ras-binding domain of Raf-1. This interaction has been shown to require GTP binding to Ras (Taylor and Shalloway, 1996). Adhesion of β_{1A} cell transfectants to fibronectin as well as engagement of endogenous integrins by fibronectin in β_{1C} stable cell lines cultured in the presence of tetracycline stimulated Ras activation (Figure 7A, top panel, lanes 1, 2, 9, and 10). Maximal activation of Ras in CHO cells in the presence of tetracycline was observed at 10 min (Figure 7A, top panel, lanes 10–12). In contrast, in the absence of tetracycline, β_{1C} expression nearly abolished Ras activation mediated by fibronectin (Figure 7A, top panel, lanes 5–8). We investigated whether Ras could overcome the β_{1C} inhibitory effect on fibronectin-mediated ERK2 activation by expressing a constitutively active form of Ras, Ras 61 (L). Transfection of β_{1C} CHO stable cell lines with constitutively active Ras 61 (L) restored fibronectin-induced ERK2 activation to the levels observed in cells transfected with vector alone and cultured in the presence of tetracycline (Figure 7B, top panel, lanes 4 and 6). These data indicate that β_{1C} inhibits the MAP kinase pathway by preventing Ras activation.

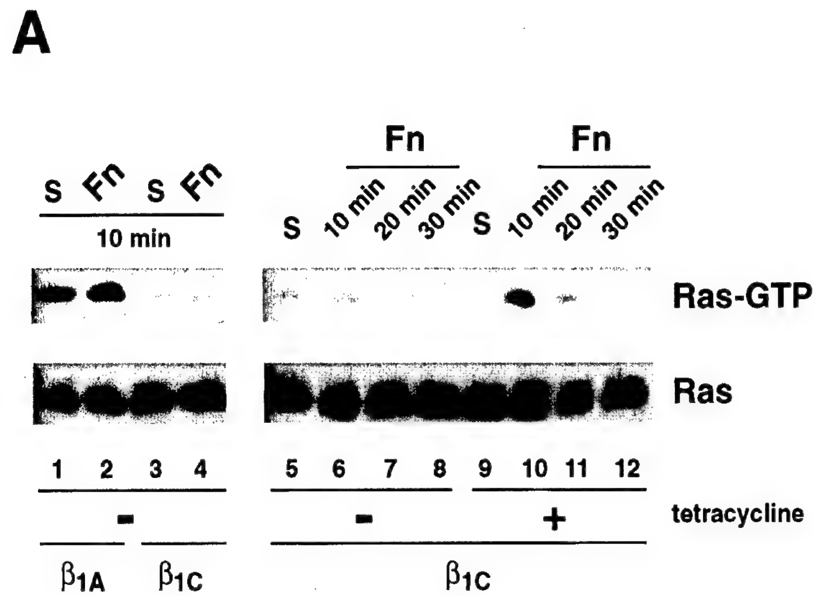
Inhibition of Cell Proliferation in β_{1C} Transfectants Is Rescued by MEK

To evaluate whether down-regulation of ERK2 activity causes inhibition of cell proliferation in β_{1C} transfectants, we transfected β_{1C} CHO stable cell lines with either MEK WT or MEK EE. The levels of expression of both MEK WT and MEK EE were comparable as determined by immunoblotting with 12CA5 mAb to hemagglutinin (our unpublished results). As expected, β_{1C} expression in CHO cells had an inhibitory effect on cell proliferation, whereas β_{1A} did not affect cell proliferation in response to serum (Figure 8A). Transfection of β_{1C} CHO stable cell lines with MEK EE restored cell proliferation to an extent similar to the level observed in cells cultured in the presence of tetracycline (Figure 8B). Thus, expression of constitutively active MEK rescues the inhibitory effect on cell proliferation exerted by β_{1C}.

DISCUSSION

In this study, as indicated in the model shown in Figure 9, we demonstrate that β_{1C} integrins inhibit ERK2 activation in response to cell adhesion to fibronectin by preventing Ras activation. It is also shown that β_{1C} inhibits Ras and ERK2 activation without affecting either FAK phosphorylation or AKT activity. Engagement of β_{1C} activates AKT but is not able to stimulate the MAP kinase pathway; this indicates that its unique cytodomain allows selective activation of the AKT kinase pathway in response to engagement of the common β_1 extracellular domain. Furthermore, constitutively active MEK restored cell proliferation in β_{1C} transfectants, suggesting that the negative effect of β_{1C} on the Ras/ERK pathway causes inhibition of cell proliferation.

The aim of this investigation was to determine the roles of two integrin variants, β_{1C} and β_{1A}, in modulating specific signaling pathways that control cell proliferation and survival. Specifically, we studied MAP kinase, FAK, and AKT pathways. MAP kinase pathway involvement in mediating cell cycle progression and gene expression, as well as the ability of FAK and AKT to support cell survival and prevent anoikis, have been well documented (Frisch *et al.*, 1996; Hungerford *et al.*, 1996; Xu *et al.*, 1996; Khwaja *et al.*, 1997; Robinson and Cobb, 1997; Downward, 1998; Guadagno and Ferrell, 1998; Brunet *et al.*, 1999; Cary and Guan, 1999). The mechanisms of integrin-mediated activation of the MAP kinase cascade comprise Ras-dependent and Ras-independent activation of ERK2 by integrins (Howe *et al.*, 1998). Our results show that, in contrast to β_{1A}, β_{1C} has an inhibitory effect on Ras and ERK2 activation mediated by fibronectin. Selective inhibition of the Ras/MAP kinase pathway by β_{1C} indicates that this integrin has the ability to either interfere with Ras membrane localization or inhibit positive regulators of Ras, or increase the activity of negative regulators of this molecule (Rebollo and Martinez-A, 1999). FAK has been shown to mediate Ras activation through Grb2/Sos binding (Schlaepfer and Hunter, 1998). However, in our system, we



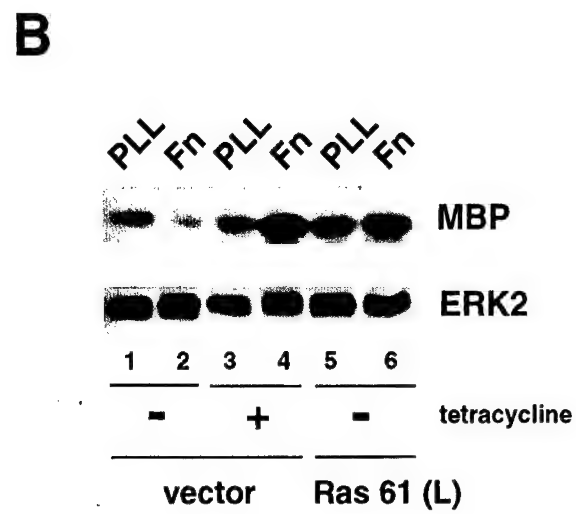


Figure 7. β_{1C} prevents Ras activation stimulated by fibronectin. (A) β_{1C} or β_{1A} CHO stable cell lines were cultured for 48 h either in the absence (lanes 1–8) or in the presence (lanes 9–12) of 1 μg/ml tetracycline and serum-starved during the last 24 h of the 48-h culture. The cells were detached and either held in suspension (S; lanes 1, 3, 5, and 9) or seeded on tissue culture plates coated with fibronectin (Fn; lanes 2, 4, 6–8, and 10–12) for 10 min (lanes 1–6, 9, and 10), 20 min (lanes 7 and 11), or 30 min (lanes 8 and 12) at 37°C. Cells were lysed, and Ras activation was analyzed by affinity precipitation with GST-RBD (top panels). Ras proteins were detected by immunoblotting with 2 μg/ml mAb to Ras (bottom panels). (B) β_{1C} CHO stable cell lines were transiently transfected with constitutively activated Ras [Ras 61 (L); lanes 5 and 6] or vector alone (vector; lanes 1–4). Cells were cultured for 48 h either in the absence (lanes 1, 2, 5, and 6) or in the presence (lanes 3 and 4) of 1 μg/ml tetracycline and starved during the last 24 h of the 48-h culture. Transfected cells were then detached and plated on dishes coated with either poly-L-lysine (PLL; lanes 1, 3, and 5) or fibronectin (Fn; lanes 2, 4, and 6) for 10 min at 37°C. ERK2 in vitro kinase activity (top panel) and expression (bottom panel) were analyzed as described for Figure 6. In A and B (bottom panels), proteins were visualized by enhanced chemiluminescence. The experiments were repeated twice with consistent results.

do not expect β_{1C} to act through FAK because β_{1C} inhibits ERK2 activity without affecting integrin signaling to FAK. This is the first description of a selective inhibitory role of the integrin cytoplasmic domain on a member of the MAP kinase family. In one instance, integrin down-regulation of FAK tyrosine phosphorylation and MAP kinase activity has been described (Sastry *et al.*, 1999). Here we show that FAK phosphorylation and AKT activation can occur in the absence of ERK2 activation, indicating that β_{1C} inhibits either a pathway downstream of FAK or AKT or a FAK- and AKT-independent pathway (Figure 9A). It has been described that PI 3-kinase is required for maximal fibronectin-mediated ERK2 activation and that it functions downstream of Ras (King *et al.*, 1997); in our β_{1C}-expressing cells, the PI 3-kinase/AKT pathway is active even though ERK2 is inhibited, suggesting that PI 3-kinase alone is not sufficient to activate ERK2 in the absence of Ras activation. It was reported recently that PKC inhibition selectively prevents ERK2 activation in response to integrin without affecting FAK tyrosine phosphorylation (Miranti *et al.*, 1999). Thus, expression of β_{1C} might down-regulate ERK2 activity in response to fibronectin adhesion via inhibition of PKC, which has been shown to act upstream of Ras (Miranti *et al.*, 1999).

The β_{1C} and β_{1A} variants have a different subcellular distribution (Meredith *et al.*, 1995); β_{1A} localizes to focal contacts, whereas β_{1C} remains diffuse on the cell surface. Thus, our results indicate that MAP kinase inhibition ob-

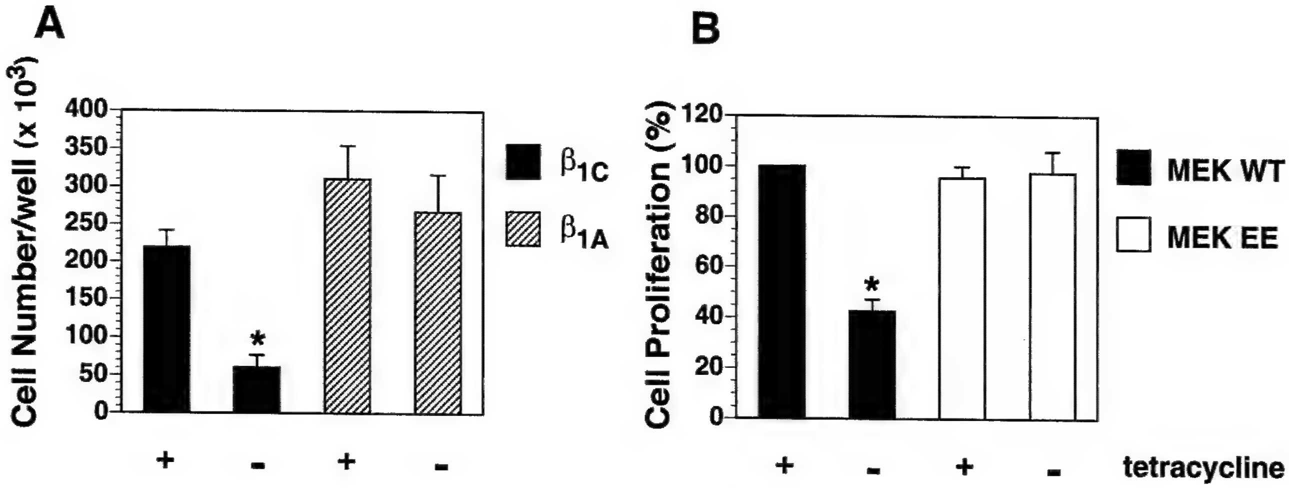


Figure 8. Constitutively activated MEK rescues β_{1C} inhibition of CHO cell proliferation. (A) β_{1C} or β_{1A} CHO stable cell lines were cultured as described for Figure 7A. Cells were detached, resuspended in serum-free medium, and plated (10,000 cells/well) on tissue culture plates coated with 1 μg/ml fibronectin for 1 h at 37°C. Attached cells were cultured for 96 h at 37°C in growth medium containing 5% FCS either in the absence or in the presence of 1 μg/ml tetracycline. Cells were washed, fixed with 3% paraformaldehyde, and stained overnight with 0.5% toluidine blue. Cell number was evaluated as described in MATERIALS AND METHODS. (B) β_{1C} CHO stable cell lines were transiently transfected with either MEK WT or MEK EE. Cells were cultured for 48 h either in the absence or in the presence of 1 μg/ml tetracycline and starved during the last 24 h of the 48-h culture. Cells were detached, plated, and cultured for 72 h, and cell number was analyzed as described for A. Cell proliferation is expressed as percent relative to the value for MEK WT cultured in the presence of tetracycline. Shown is the average ± SEM from two separate experiments. Group differences were compared with one-way analysis of variance. In A, the differences in proliferation either between β_{1C} CHO stable cell lines in the absence (*) and in the presence of tetracycline or between β_{1C} CHO stable cell lines cultured in the absence of tetracycline (*) and β_{1A} CHO stable cell lines cultured either in the presence or in the absence of tetracycline are statistically significant ($p < 0.05$). In B, the differences in cell proliferation either between MEK WT in the absence of tetracycline (*) and in the presence of tetracycline or between MEK WT in the absence of tetracycline (*) and MEK EE cultured either in the presence or in the absence of tetracycline are statistically significant ($p < 0.05$).

served in β_{1C} transfectants does not require β_{1C} recruitment to focal adhesion complexes. In a previous report, we had attempted to study ERK2 activation in response to β_{1C} or β_{1A} engagement by TS2/16. However, we had not detected either β_{1C} or β_{1A} integrin-mediated ERK2 activation because of the low integrin levels and the low number of cells transfected in the transient expression system (Fornaro *et al.*, 1999). Here, using stable cell lines that have higher levels of expression, we show the failure of β_{1C} to activate ERK2, although we detect MAP kinase activation in response to β_{1A} engagement (Figure 9B). In this study, it is also shown that AKT phosphorylation is observed in response to β_{1C} engagement (Figure 9B). Therefore, specific domains in the extreme carboxy-terminal region of β_1 are not required to activate the PI 3-kinase/AKT pathway. In our cell system as well as in the cell systems of others (King *et al.*, 1997), AKT activation is PI 3-kinase dependent, because wortmannin completely prevents AKT serine phosphorylation in response to either endogenous or exogenous integrin engagement. Ras is a potent activator of PI 3-kinase, in addition to Raf and non-Raf pathways (Rebollo and Martinez-A, 1999); thus, in our experimental system, in which Ras is inhibited, stimulators of PI 3-kinase different from Ras are expected to be active. FAK is a potential candidate; PI 3-kinase is activated by FAK (Chen *et al.*, 1996a). In our system, a causal effect of FAK activation on PI 3-kinase/AKT pathway stimulation, in response to either β_{1C} or β_{1A} engagement, remains to be investigated. Recent evidence points also to integrin-linked kinase (ILK) as a candidate effector for activation of AKT in response to integrin engagement, because ILK mediates PI 3-kinase–dependent AKT activation and binds the integrin β_1 cytodomain (Hanningan *et al.*, 1996; Delcommenne *et al.*, 1998). However, ILK binds the integrin β_1 cytodomain in a region that is not found in β_{1C} (S. Dedhar, personal communication). Thus, although it is crucial for signaling pathways activated in response to β_{1A} ligation, ILK is unlikely to play a role in the activation of AKT in β_{1C} transfectants.

Cell adhesion to fibronectin or to β_1 ligands is unaffected in response to β_{1C} expression. Furthermore, the β_{1C} variant associates with the same α subunits as β_{1A}, indicating that up-regulation of β_{1C} allows the cell to preserve the interaction with the extracellular matrix but, at the same time, to inhibit cell cycle progression. Therefore, we suggest that by expressing variant β_1 intracellular domains, cells may accomplish the delicate task of inhibiting proliferation without affecting either selective downstream survival signals (FAK and AKT) mediated by integrins or interactions with the extracellular environment. This observation is very important because in vivo, β_{1C} is expressed in nonproliferative and differentiated epithelium (Fornaro *et al.*, 1998) and is selectively down-regulated in prostatic adenocarcinoma (Fornaro *et al.*, 1996). Thus, the ability of β_{1C} to sustain activation of signals that stimulate survival and differentiation (FAK and AKT) (Downward, 1998; Jiang *et al.*, 1999) might be crucial to preventing apoptosis while blocking cell cycle progression and maintaining a differentiated phenotype. Failure to maintain a differentiated phenotype is be-

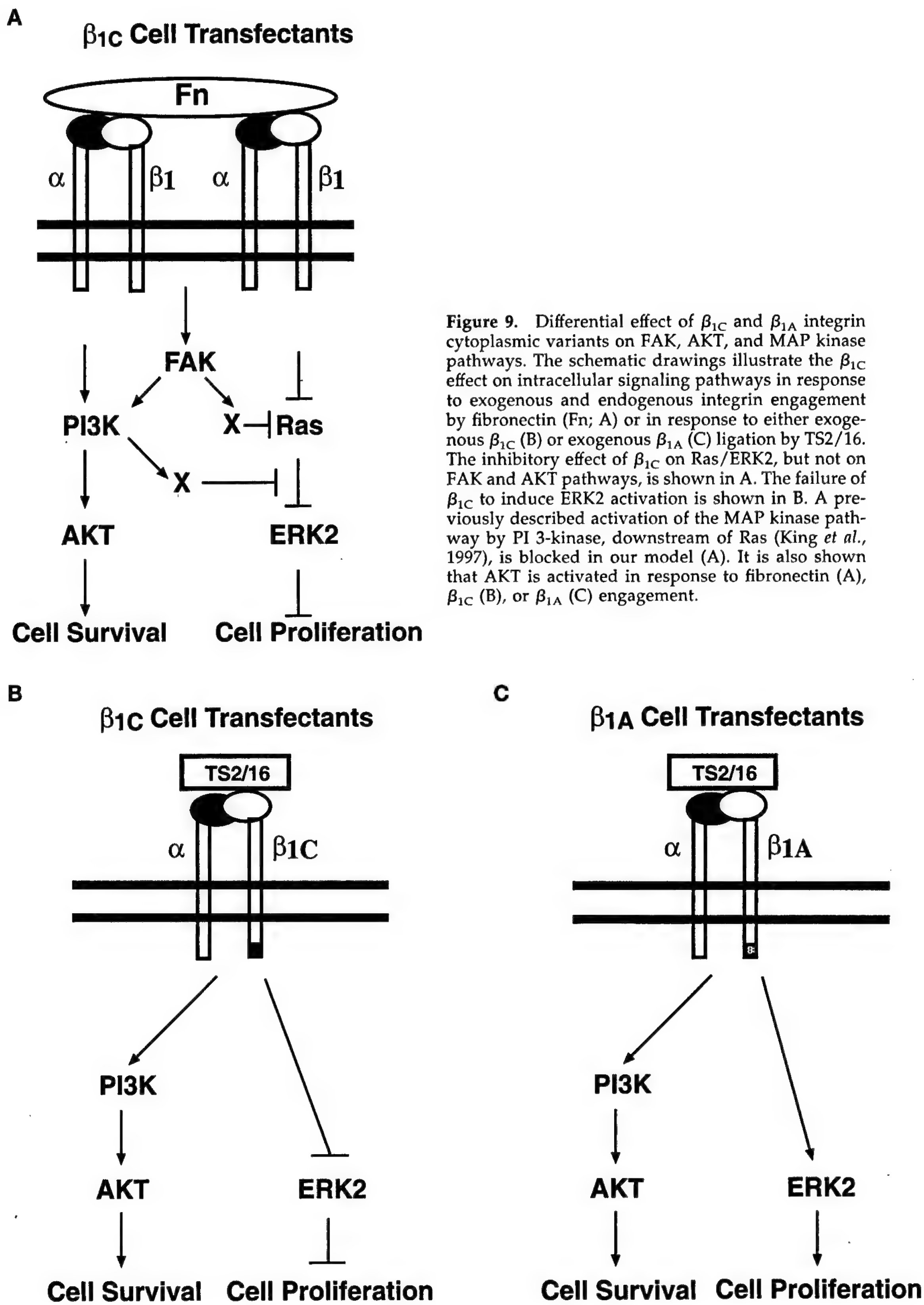


Figure 9. Differential effect of β_{1C} and β_{1A} integrin cytoplasmic variants on FAK, AKT, and MAP kinase pathways. The schematic drawings illustrate the β_{1C} effect on intracellular signaling pathways in response to exogenous and endogenous integrin engagement by fibronectin (Fn; A) or in response to either exogenous β_{1C} (B) or exogenous β_{1A} (C) ligation by TS2/16. The inhibitory effect of β_{1C} on Ras/ERK2, but not on FAK and AKT pathways, is shown in A. The failure of β_{1C} to induce ERK2 activation is shown in B. A previously described activation of the MAP kinase pathway by PI 3-kinase, downstream of Ras (King *et al.*, 1997), is blocked in our model (A). It is also shown that AKT is activated in response to fibronectin (A), β_{1C} (B), or β_{1A} (C) engagement.

lieved to be an early event in cancer progression (Hunter, 1997), suggesting that loss of β_{1C} might activate a cascade that contributes to a transformed phenotype.

We have shown previously that β_{1C} expression increases p27^{kip1} protein levels (Fornaro *et al.*, 1998). This cyclin kinase inhibitor is highly expressed in nonproliferative, quiescent cells, and its forced overexpression is sufficient to inhibit cell proliferation (Sherr and Roberts, 1995) and apoptosis (Hiromura *et al.*, 1999). In prostate cancer, loss of p27^{kip1} is an adverse prognostic factor that correlates with poor patient survival (Catzavelos *et al.*, 1997; Loda *et al.*, 1997; Porter *et al.*, 1997; Tsihlias *et al.*, 1998; Yang *et al.*, 1998). A report has indicated that oncogenic Ras-induced degradation of p27^{kip1} occurs through activation of the MAP kinase cascade (Kawada *et al.*, 1997). Thus, it is conceivable that by blocking Ras activation, β_{1C} expression, at variance with β_{1A} expression, achieves the goal of inhibiting ERK2 activation and, consequently, p27^{kip1} degradation and cell proliferation.

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Substrate Specificity of $\alpha_v\beta_3$ Integrin-mediated Cell Migration and Phosphatidylinositol 3-Kinase/AKT Pathway Activation*

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The $\alpha_v\beta_3$ integrin has been shown to bind several ligands, including osteopontin and vitronectin. Its role in modulating cell migration and downstream signaling pathways in response to specific extracellular matrix ligands has been investigated in this study. Highly invasive prostate cancer PC3 cells that constitutively express $\alpha_v\beta_3$ adhere and migrate on osteopontin and vitronectin in an $\alpha_v\beta_3$-dependent manner. However, exogenous expression of $\alpha_v\beta_3$ in noninvasive prostate cancer LNCaP (β_3-LNCaP) cells mediates adhesion and migration on vitronectin but not on osteopontin. Activation of $\alpha_v\beta_3$ by epidermal growth factor stimulation is required to mediate adhesion to osteopontin but is not sufficient to support migration on this substrate. We show that $\alpha_v\beta_3$-mediated cell migration requires activation of the phosphatidylinositol 3-kinase (PI 3-kinase)/protein kinase B (PKB/AKT) pathway since wortmannin, a PI 3-kinase inhibitor, prevents PC3 cell migration on both osteopontin and vitronectin; furthermore, $\alpha_v\beta_3$ engagement by osteopontin and vitronectin activates the PI 3-kinase/AKT pathway. Migration of β_3-LNCaP cells on vitronectin also occurs through activation of the PI 3-kinase pathway; however, AKT phosphorylation is not increased upon engagement by osteopontin. Furthermore, phosphorylation of focal adhesion kinase (FAK), known to support cell migration in β_3-LNCaP cells, is detected on both substrates. Thus, in PC3 cells, $\alpha_v\beta_3$ mediates cell migration and PI 3-kinase/AKT pathway activation on vitronectin and osteopontin; in β_3-LNCaP cells, $\alpha_v\beta_3$ mediates cell migration and PI 3-kinase/AKT pathway activation on vitronectin, whereas adhesion to osteopontin does not support $\alpha_v\beta_3$-mediated cell migration and PI 3-kinase/AKT pathway activation. We conclude therefore that $\alpha_v\beta_3$ exists in multiple functional states that can bind either selectively vitronectin or both vitronectin and osteopontin and that can differentially activate cell migration and intracellular signaling pathways in a ligand-specific manner.

Integrins are heterodimeric cell surface receptors that consist of noncovalently associated α and β subunits; these receptors have been shown to play a role in cell migration, proliferation, and gene transcription and can affect cancer cell invasion and growth (1–3). The role of $\alpha_v\beta_3$ integrin in mediating cell migration and survival has been described (4–6). Exogenous expression of $\alpha_v\beta_3$ has been shown to increase melanoma tumor growth and metastases (7, 8), to induce conversion from radical to vertical growth phase in primary human melanoma cells, and to promote melanoma cell survival *in vivo* (9) and in three-dimensional collagen gels (10) indicating $\alpha_v\beta_3$ contribution at the level of motility and proliferation *in vivo*. We have previously shown that highly invasive and metastatic human PC3 prostate cancer cells express $\alpha_v\beta_3$ whereas nontumorigenic and noninvasive LNCaP cells do not (5).

The $\alpha_v\beta_3$ integrin is a promiscuous receptor that mediates adhesion of several cell types to different ligands and of cancer cells to platelets (11, 12). Among others, vitronectin (VN)[1]- (11, 13, 14) and osteopontin (OPN)-coated (15–20) substrates have been shown to support cell adhesion via $\alpha_v\beta_3$.

OPN is expressed in mature bone where prostate cancer cells preferentially metastasize. A causal role for OPN during tumor progression has been suggested by several studies, including the observation that high levels of OPN support a tumorigenic and metastatic phenotype (21, 22). OPN is up-regulated in prostate cancer and other carcinomas (23, 24) and increases anchorage-independent growth of prostate cancer cells (22) as well as proliferation of normal prostate cells (25). Furthermore, it is found in plasma of patients with metastatic diseases, and it increases metastatic ability of transformed cells (26, 27). Its interaction with different surface receptors has been shown: specifically, $\alpha_4\beta_1$, $\alpha_8\beta_1$, $\alpha_9\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_5$, and CD44 on different cell types (28–35). The ability of OPN to support haptotaxis of different cell types via $\alpha_v\beta_3$ has been shown (16). However, the signaling mechanisms activated via OPN-$\alpha_v\beta_3$ interaction that support cell migration have never been described.

It has recently been shown that $\alpha_v\beta_3$ can be activated (36–38) in a cell-type specific (39) manner. Its activation appears to be a sophisticated mechanism to induce adhesion to $\alpha_v\beta_3$ ligands, specifically to prothrombin via protein kinase C activation or ADP stimulation, to VN via either hepatocyte growth factor or AP5, an antibody to $\alpha_v\beta_3$, and to OPN via either AP5 or agonists, including ADP (38, 40–42). It has been recently shown that activated $\alpha_v\beta_3$ mediates cell adhesion and migration to bone sialoprotein (43). In one instance, upon activation by AP5, $\alpha_v\beta_3$ was shown to increase adhesion and migration of $\alpha_v\beta_3$-expressing melanoma cells on OPN and VN in a comparable manner (41). However, the role of activation-dependent

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[1] The abbreviations used are: VN, vitronectin; OPN, osteopontin; FN, fibronectin; PI 3-kinase, phosphatidylinositol 3-kinase; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; FACS, fluorescence-activated cell sorter; FAK, focal adhesion kinase; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; EGF, epidermal growth factor; HER, human EGF receptor; PBS, phosphate-buffered saline.

and activation-independent ligands of $\alpha_v\beta_3$ in modulating cell functions and downstream signaling events has not been described.

Several signaling molecules, specifically FAK, PI 3-kinase, and members of the MAP kinase family, play a role in modulating integrin-mediated cell migration (44). FAK is a nonreceptor tyrosine kinase localized in focal contacts that becomes tyrosine-phosphorylated and subsequently activated upon integrin-mediated cell adhesion to several matrix proteins, including VN (5, 45, 46). FAK phosphorylation of tyrosine 397 (Tyr^{397}) is crucial for cell migration (47). PI 3-kinase is a lipid kinase involved in proliferation, survival, and migration in response to growth factors including EGF and integrin signaling (48–50). PI 3-kinase forms a complex with FAK via FAK-Tyr^{397} in response to cell adhesion or platelet-derived growth factor stimulation (51, 52), and it is known to act as a downstream effector of FAK and to control FAK-induced cell migration activated by cell adhesion to extracellular matrix proteins (53, 54). AKT plays an important role in transducing survival signals in response to several growth factors and β_1 integrin engagement (55, 56) and very recently in supporting vascular endothelial growth factor-induced chemotaxis (57). In response to integrin engagement, AKT activation is PI 3-kinase-dependent because wortmannin completely prevents AKT serine phosphorylation (50) and is also controlled by Cdc42, a member of the GTPase family (58) or by ILK (59). Integrin engagement has also been shown to stimulate activation of two members of the MAP kinase family, extracellular signal-regulated kinase-1 and -2 (ERK1/2) (2), which contribute to integrin-mediated cell migration (60, 61).

In this study, we show that adhesion of invasive prostate cancer PC3 cells to OPN and VN activates the PI 3-kinase/AKT signaling pathway; however, exogenous expression of $\alpha_v\beta_3$ in noninvasive LNCaP cells mediates VN binding but requires EGF stimulation to mediate binding to OPN. In these cells, adhesion to OPN does not support cell migration and PI 3-kinase/AKT pathway activation, whereas $\alpha_v\beta_3$ mediates cell migration and PI 3-kinase/AKT pathway activation on VN. These results show that $\alpha_v\beta_3$ is expressed in multiple functionally different states and is able to mediate cell migration in a substrate-specific and functional state-dependent manner; finally, they show that $\alpha_v\beta_3$ activates intracellular signaling pathways in a selective manner in response to individual ligands.

EXPERIMENTAL PROCEDURES

Cell Lines and Materials—LNCaP stable transfectants expressing β_3 (β_3-LNCaP) and mock transfectants (mock-LNCaP) have been described (5). PC3 and LNCaP cells were cultured in RPMI 1640, A431 (American Type Culture Collection (ATCC), Manassas, VA), and HeLa S3 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing either 10 or 5% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 292 μg/ml L-glutamine (all from Gemini Bio-Products, Inc., Calabasas, CA), 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (Life Technologies, Inc.). Monoclonal antibody against $\alpha_v\beta_3$ integrin LM609 was used as ascites (provided by Dr. D. A. Cheresh, The Scripps Research Institute, La Jolla, CA) or as affinity-purified IgG (Chemicon International, Inc., Temecula, CA). Monoclonal antibodies against $\alpha_v\beta_5$ integrin were: P1F6, used as ascites and purchased from Life Technologies, Inc., and P3G2 hybridoma supernatant, a gift of Dr. E. A. Wayner (Fred Hutchinson Cancer Research Center, Seattle, WA (62)). Monoclonal antibody to β_1, TS2/16 hybridoma supernatant, was purchased from ATCC. Monoclonal antibody against human EGF receptor, HER (EGFR-528) and polyclonal antibody against ERK1/2 (K-23) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Negative control 1C10 ascites and X653 hybridoma supernatant have been described (5). Polyclonal antibodies against phospho-AKT (Ser^{473}) and AKT, as well as monoclonal antibody against phospho-ERK1/2 Thr^{202}/Tyr^{204} (E10) were purchased from New England Biolabs, Inc. (Beverly, MA). Fibronectin (FN) was purified from human plasma, and VN was purified from human serum as described previously (63, 64). Laminin-1 was purchased from Life Technologies, Inc. OPN purified from human milk was a gift from Dr. D. R. Senger (Beth Israel Deaconess Medical Center, Boston, MA (65)). Bovine serum albumin (BSA) and dimethyl sulfoxide (Me_2SO) were purchased from Sigma. Recombinant human EGF was purchased from R&D Systems (Minneapolis, MN). Wortmannin was purchased from Calbiochem.

Flow Cytometric Analysis—Fluorescence-activated cell sorter (FACS) analysis was performed using LM609 ascites (1:500), P1F6 ascites (1:500), TS2/16 hybridoma supernatant (1:10), or EGFR-528 (5 μg/ml) described above. The 1C10 (1:500), X653 (1:10), and nonimmune mouse IgG (5 μg/ml; Cappel, Durham, NC) were used as negative controls. After washing the primary antibodies with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG (40 μg/ml; Cappel) at 4 °C for 30 min. The cells were washed again with PBS. FACS sorting were performed using a FACSort, and analysis was performed using CellQuest 2.0 (Becton Dickinson, Mountain View, CA).

Cell Adhesion and Migration Assays—Cell adhesion and migration assays have been described previously (5). Cell adhesion assays were performed by incubating cells with the coated substrates at 37 °C for 1 or 3 h, and quantitated by measuring absorbance at 630 nm using a Titertek Multiskan Bichromatic (ICN Pharmaceuticals, Inc., Costa Mesa, CA) for crystal violet staining. In some experiments, adhesion was quantitated using cells labeled with [^{51}Cr]sodium chromate (Amersham Pharmacia Biotech). The quantitation of [^{51}Cr]sodium chromate-labeled cells was carried out by β-counting, and the counts/min were converted to cell numbers based on cell labeling efficiency. Cell migration assays was performed by incubating cells with the coated Transwell chamber (12-μm pore size, Costar, Cambridge, MA) at 37 °C for 4 h. Cell adhesion and migration assays with EGF were performed using cells that had been preincubated with 200 ng/ml EGF at 4 °C for 60 min; EGF was present at the same concentration during the experiments. In the wortmannin inhibition assays, cells were harvested using trypsin (0.05%)/EDTA (0.53 mM) following neutralization in an equal volume of 0.5 mg/ml soybean trypsin inhibitor and washed twice in PBS. Wortmannin dissolved in Me_2SO at a stock concentration of 10 mM and further diluted to the indicated concentrations in PBS was added to the cell suspension at the time of cell seeding onto the coated plates. Wortmannin was not preincubated with the cells before addition to the coated wells. Cell adhesion and migration were carried out in the presence of the indicated amounts of wortmannin. After incubation at the indicated times, unbound cells were washed away using PBS. The adherent or migrated cells were fixed using 3% paraformaldehyde at 4 °C for 30 min followed by crystal violet staining at 25 °C for 3 h. In the case of adhesion experiments using ^{51}Cr-labeled cells, bound cells were lysed in the plate without fixation or staining. Triplicate observations were performed.

AKT, Phospho-AKT, FAK, and Phospho-FAK Immunoblotting—Cells were starved in serum-free RPMI 1640 medium for 24 h, detached using 0.05% trypsin, 0.53 mM EDTA, and neutralized with 0.5 mg/ml soybean trypsin inhibitor. The cells were washed twice with serum-free RPMI 1640, resuspended in the same medium, and incubated with either 200 ng/ml EGF or serum-free medium at 4 °C for 60 min. Cells were either held in suspension using 10 mg/ml BSA-coated plate or seeded on 60-mm dishes coated with FN, VN, or OPN at indicated concentrations and allowed to attach at 37 °C for the indicated times. Cells were lysed in 1% Nonidet P-40 lysis buffer: 50 mM Tris, pH 7.5 (American Bioanalytical, Natick, MA), 1% Nonidet P-40 (Calbiochem), 50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, 50 mM NaF, 0.2 mM EGTA, 1 mM EDTA, pH 8.0 (all from Sigma). The protein concentration of each lysate was determined using BCA protein assay reagent (Pierce). For AKT and phospho-AKT immunoblotting, 30 μg of cell lysates were loaded in each lane on a 7.5% or 10% SDS-PAGE under reducing conditions. The proteins were transferred to polyvinylidene difluoride membranes (Millipore). The membrane was blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20 at 25 °C for 3 h. Polyclonal antibodies to AKT (0.1 μg/ml) or to phospho-AKT Ser^{473} (0.05 μg/ml) were incubated with the membrane at 4 °C for 16 h. Then, the membrane was incubated with peroxidase-coupled anti-rabbit IgG at 25 °C for 1 h. The specific proteins were detected with enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). Quantitative analysis was performed using a computing densitometer (Molecular Dynamics, Sunnyvale, CA).

Analysis of FAK phosphotyrosine content was performed as described previously (5). Briefly, precleared lysates were prepared as above and then immunoprecipitated using 0.5 μg of polyclonal antibody

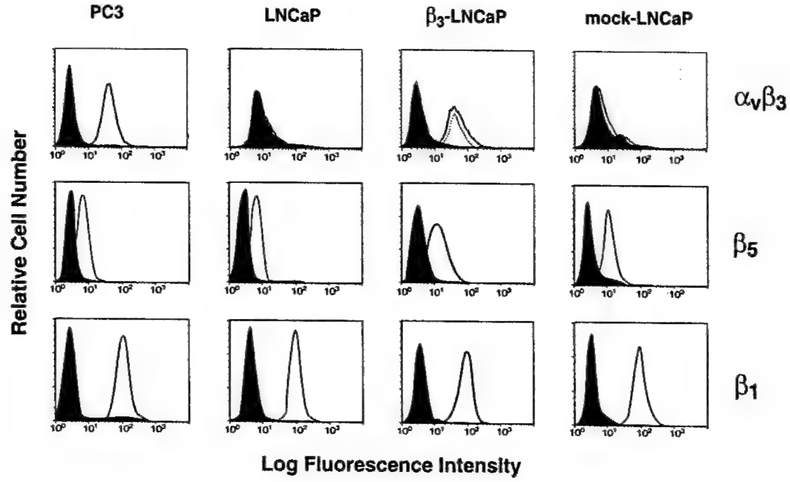


FIG. 1. **Expression of integrins in PC3, parental LNCaP, β_3-LNCaP and mock-LNCaP cells.** FACS analysis was performed as described under "Experimental Procedures" using LM609 (1:500), a monoclonal antibody to $\alpha_v\beta_3$ (*top* row); P1F6 (1:500), a monoclonal antibody to $\alpha_v\beta_5$ (*middle* row); or TS2/16 (1:10), a monoclonal antibody to β_1 (*bottom* row). Secondary anti-mouse IgG is conjugated to fluorescein isothiocyanate. 1C10 (1:500) or nonspecific hybridoma supernatant X653 (1:10) were used as negative control antibodies (*filled profiles*). FACS analysis of $\alpha_v\beta_3$ expression in β_3-LNCaP cells (*third profile* of *top* row) is shown in the presence (*dotted line*) or absence (*continuous line*) of EGF.

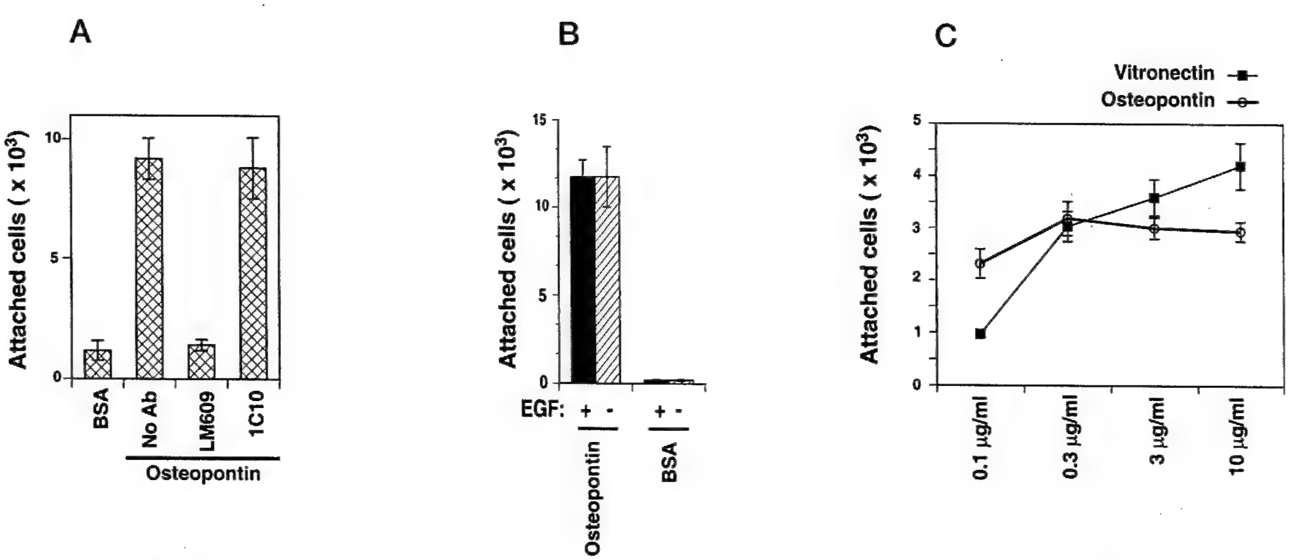


FIG. 2. **PC3 cells adhere to OPN in an $\alpha_v\beta_3$-dependent manner.** *A*, adhesion of ^{51}Cr-labeled PC3 cells to OPN (10 μg/ml) was performed in the presence of LM609 (1:100), a monoclonal antibody to $\alpha_v\beta_3$, or 1C10 (1:100) as negative control. *No Ab* represents cell adhesion to OPN in the absence of an antibody. Cell adhesion to BSA (10 mg/ml) was used as negative control. The difference between adhesion to OPN in the presence of LM609 or of 1C10 is statistically significant ($p < 0.01$). The experiment was repeated three times with consistent results. *B*, ^{51}Cr-labeled PC3 cell adhesion to 10 μg/ml OPN in the presence (*solid bars*) or absence (*hatched bars*) of 200 ng/ml EGF is shown. Cell attachment to BSA (10 mg/ml) in the presence or absence of 200 ng/ml EGF was used as negative control. *C*, the number of ^{51}Cr-labeled PC3 cells attached to 96-well plates coated with the indicated concentrations of VN (*solid*) or OPN (*open*) at 37 °C for 2 h in the absence of EGF is shown. *Error bars*, mean ± S.E. ($n = 3$).

to FAK, C-20 (Santa Cruz Biotechnology, Inc). Immunoblotting analysis was performed using 1 μg/ml anti-phosphotyrosine monoclonal antibody, PY20 (Transduction Laboratories, San Diego, CA). To detect immunoprecipitated proteins, membranes were stripped and reblotted using C-20 (0.1 μg/ml). Experiments were repeated three times. Quantitative analysis was performed using a computing densitometer.

ERK1/2 and Phospho-ERK1/2 Immunoblotting—Immunoblotting was performed as described previously (66). Cells were harvested as described before. Cells were washed once with 0.5 mg/ml soybean trypsin inhibitor and twice with serum-free RPMI 1640 medium. Cells were resuspended in serum-free RPMI 1640 medium, incubated at 37 °C without CO_2 with agitation for 15 to 30 min, and plated on 60-mm Petri dishes that had been coated with 3 μg/ml either VN or FN. The concentration of human VN and FN used for coating had been previously determined to generate comparable cell attachment (data not shown). Cells were incubated at 37 °C with 5% CO_2 for the indicated intervals. The attached cells were washed twice with PBS and lysed in 25 mM HEPES, pH 7.6, 0.1% Triton X-100, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, 0.2 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine. Thirty μg of each lysate were loaded on 10% SDS-PAGE (9.93% acrylamide, 0.07% bisacrylamide) under reducing conditions, and proteins were transferred to polyvinylidene difluoride membranes. After blocking, the membrane was incubated with 0.1 μg/ml polyclonal antibody to ERK1/2 (Santa Cruz Biotechnology, Inc.) or monoclonal antibody to phospho-ERK1/2 Thr^{202}/Tyr^{204} (1:2000) at 4 °C for 16 h. The membranes were washed using 0.1% Tween 20 in Tris-buffered saline and incubated with a peroxidase-conjugated goat affinity-purified antibody to rabbit IgG at room temperature for 1 h. ERK1/2 or phosphorylated ERK1/2 was detected using enhanced chemiluminescence.

Statistical Analysis—Statistical analysis was performed using the Student's *t* test or one way analysis of variance, Sigma Stat (Jandel Scientific, San Rafael, CA).

RESULTS

EGF Mediates Cell Adhesion to OPN via $\alpha_v\beta_3$—Highly metastatic PC3 and nonmetastatic LNCaP prostate cancer cells differentially express the $\alpha_v\beta_3$ integrin but express comparable levels of β_5 and β_1 (Fig. 1 and Ref. 5). To investigate whether $\alpha_v\beta_3$ mediated prostate cancer cell adhesion to OPN, we analyzed the ability of PC3 cells and LNCaP cells stably transfected with β_3 cDNA (β_3-LNCaP) to bind OPN. PC3 cells adhered to OPN (Fig. 2, *A* and *B*) in an $\alpha_v\beta_3$-dependent manner, because LM609, a monoclonal antibody to $\alpha_v\beta_3$, inhibited their adhesion to OPN (Fig. 2*A*). PC3 were shown to attach equally

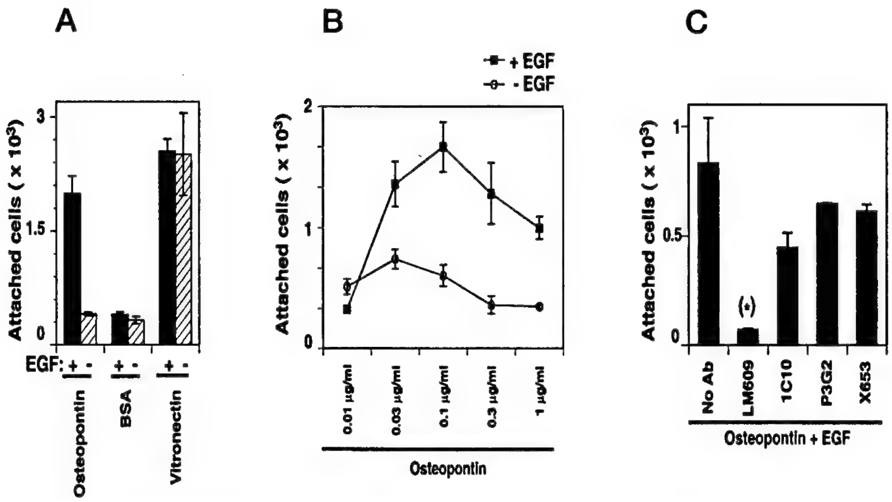


FIG. 3. **Adhesion of β_3-LNCaP transfectants to OPN requires activation of $\alpha_v\beta_3$ by EGF.** *A*, ^{51}Cr-labeled β_3-LNCaP transfectants attached to OPN (10 μg/ml), VN (3 μg/ml) in the presence (*solid bars*) or absence (*hatched bars*) of EGF (200 ng/ml) are shown. BSA (10 mg/ml) with and without EGF is shown as negative control. *B*, β_3-LNCaP cell adhesion to the indicated concentrations of OPN in the presence (*solid*) or absence (*open*) of 200 ng/ml EGF at 37 °C for 3 h is shown. Except for one concentration (0.01 μg/ml), the differences between cell adhesion in the presence or absence of EGF at each coating concentration of OPN were statistically significant ($p < 0.05$). *C*, adhesion of β_3-LNCaP transfectants to OPN in the presence of EGF was performed in the presence of LM609 (1:100 ascites; *, $p < 0.05$) antibody to $\alpha_v\beta_3$ or P3G2 antibody to $\alpha_v\beta_5$ (1:5 culture supernatant). 1C10 (1:100 ascites) and X653 (1:5 culture supernatant) were used as negative controls. *No Ab* represents cell adhesion to OPN with EGF in the absence of an antibody. All experiments were repeated at least three times with consistent results. *Error bars*, mean ± S.E. (n = 3).

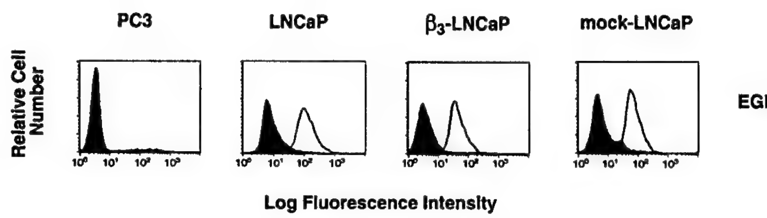


FIG. 4. **Expression of HER in PC3, parental LNCaP, β_3-LNCaP, and mock-LNCaP cells.** HER expression was measured by FACS analysis as described under "Experimental Procedures" using EGFR-528 (5 μg/ml), a monoclonal antibody to HER. Nonimmune IgG (5 μg/ml) were used as negative control (*filled profiles*).

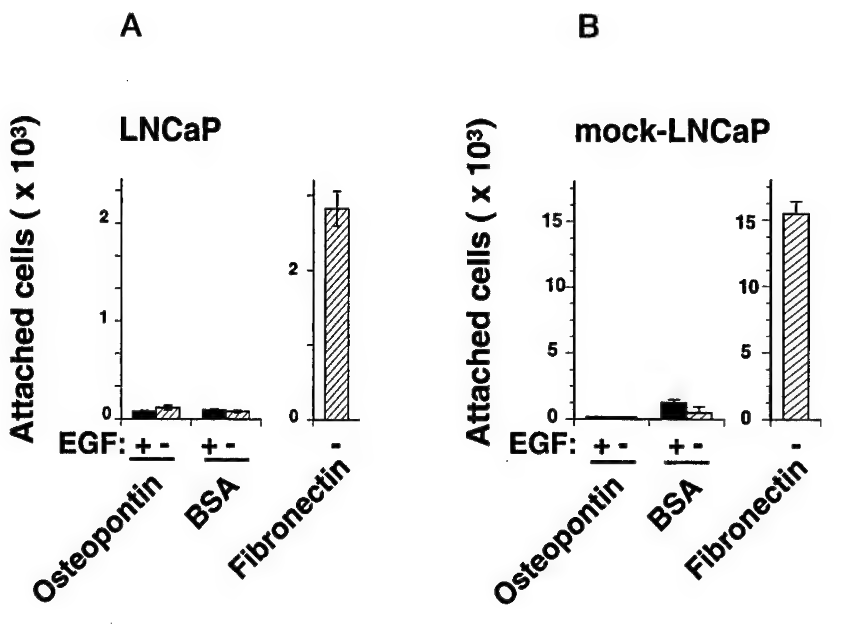


FIG. 5. **Adhesion of parental LNCaP and mock-LNCaP cells to OPN is not increased in the presence of EGF.** *A*, ^{51}Cr-labeled parental LNCaP cell attachment to OPN (10 μg/ml) either in the presence (*solid bars*) or in the absence (*hatched bars*) of 200 ng/ml EGF is shown. *B*, ^{51}Cr-labeled mock-LNCaP cell attachment to OPN (10 μg/ml) either in the presence (*solid bars*) or in the absence (*hatched bars*) of 200 ng/ml EGF is shown. Cell attachment to FN (3 μg/ml) is shown as control of cell adhesion. Cell attachment to BSA (10 mg/ml) in the presence or absence of 200 ng/ml EGF is shown as a negative control. All experiments were repeated at least two times with consistent results. *Error bars*, mean ± S.E. (n = 3).

well to OPN and VN, although at the lowest concentration tested (0.1 μg/ml), OPN was more active in supporting cell adhesion than VN (Fig. 2*C*). Surprisingly, β_3-LNCaP cells did not bind OPN (Fig. 3, *A* and *B*), although a significant amount of $\alpha_v\beta_3$ was expressed on the cell surface (Fig. 1), and the cells did adhere to VN in an $\alpha_v\beta_3$-dependent manner (Fig. 3*A*) (5). We hypothesized that exogenously expressed $\alpha_v\beta_3$ was in a conformation that did not bind OPN and that external stimuli would be required for its activation. It has been shown that $\alpha_v\beta_3$ activation requires protein kinase C (38) and that EGF and its receptor activate protein kinase C (67, 68). Thus, we tested the ability of EGF to activate $\alpha_v\beta_3$. EGF increased β_3-LNCaP cell adhesion to OPN but had no effect on BSA (Fig. 3*A*). EGF stimulation did not increase $\alpha_v\beta_3$ expression levels in β_3-LNCaP cells (Fig. 1). In contrast, in the presence of EGF, PC3 cell adhesion to OPN was not increased (Fig. 2*B*). EGF-

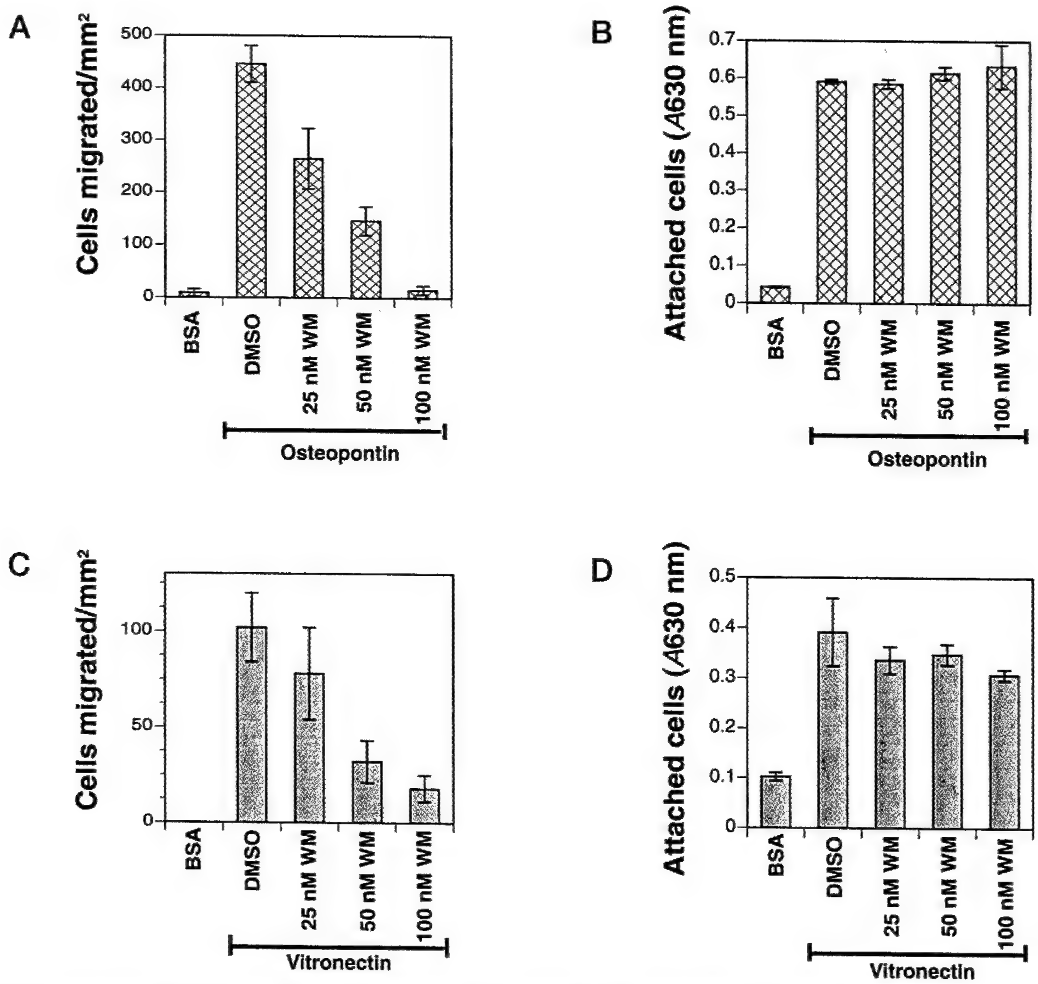


FIG. 6. **PC3 cell migration on OPN and VN is mediated by PI 3-kinase.** Cell migration was performed at 37 °C for 4 h as shown under "Experimental Procedures" using Transwell chambers coated on both sides with BSA (10 mg/ml), VN (3 μg/ml), and OPN (10 μg/ml). *A*, migration of PC3 cells on OPN in the presence of the indicated concentrations of wortmannin (*WM*) at 37 °C for 4 h is shown. *B*, adhesion of PC3 cells to OPN (10 μg/ml) in the presence of indicated concentrations of wortmannin at 37 °C for 2 h is shown. *C*, migration of PC3 cells on VN in presence of the indicated concentrations of wortmannin at 37 °C for 4 h is shown. *D*, adhesion of PC3 cells to VN (10 μg/ml) in the presence of wortmannin at 37 °C for 2 h is shown. In *panels A–D,* migration and adhesion of PC3 cells to BSA (10 mg/ml) is shown as negative control; Me_2SO (*DMSO*) was used as vehicle for wortmannin. In *panels B* and *D*, attached cells were fixed in 3% paraformaldehyde at 4 °C for 30 min, stained with 0.5% crystal violet at room temperature for at least 2 h, and described under "Experimental Procedures." Triplicate observations were performed. The numbers of migrated cells/mm² are shown. All experiments were repeated at least three times with consistent results. *A–D, error bars*, mean ± S.E. (n = 3).

stimulated β_3-LNCaP cell adhesion to OPN was blocked by LM609 but not by P3G2, an antibody to $\alpha_v\beta_5$ (Fig. 3*C*). P3G2 previously shown to block $\alpha_v\beta_5$ adhesion to its ligand was active in inhibiting HeLa cell adhesion to VN (data not shown) at the concentrations used in Fig. 3*C*.

β_3-LNCaP and mock-LNCaP transfectants express HER at comparable levels (Fig. 4); however, the effect of EGF was specific for β_3 because OPN adhesion was not up-regulated either in parental LNCaP cells (Fig. 5*A*) or in mock-transfected LNCaP cells (Fig. 5*B*) that do not express $\alpha_v\beta_3$. In conclusion, EGF is required to activate $\alpha_v\beta_3$ adhesion of noninvasive prostate cancer LNCaP cells to OPN, indicating a new level of complexity in the regulation of cell adhesion by $\alpha_v\beta_3$.

Role of PI 3-Kinase in $\alpha_v\beta_3$-mediated Cell Migration—To investigate whether adhesion to OPN would result in increased cell migration, PC3 and β_3-LNCaP cells were analyzed in Transwell migration assays using an EGF concentration gradient or a constant concentration of EGF in both the upper and bottom chambers. PC3 cells migrated on both OPN and VN substrates; migration on OPN occurred at a higher extent than on VN (Fig. 6, *A* and *C*). Wortmannin, a PI 3-kinase inhibitor, inhibited PC3 cell migration on OPN and VN (Fig. 6, *A* and *C*) but not adhesion on these substrates (Fig. 6, *B* and *D*). Instead, β_3-LNCaP cells did not migrate on OPN in the presence or absence of EGF (Fig. 7*A* and data not shown), although these cells migrated on FN (Fig. 7*A*) and VN (Fig. 7*B* and Ref. 5). Wortmannin inhibited β_3-LNCaP cell migration on VN (Fig. 7*B*) but not adhesion to VN (Fig. 7*C*). In all experiments, concentrations of VN and OPN that gave comparable levels of adhesion were selected; OPN coating of either the bottom part or of both sides of the Transwell chamber gave comparable results (data not show). EGF was active in mediating chemotaxis of A431 cells on laminin-1-coated substrates (data not shown). These results show that a signaling step, crucial for cell migration, failed to be activated in β_3-LNCaP cell transfectants adherent to OPN but was active in PC3 cells.

Substrate Specificity of PI 3-Kinase Pathway Activation—To analyze whether a differential activation by $\alpha_v\beta_3$ of downstream integrin-mediated signaling events occurs in response to adhesion to a specific substrate, PC3 and β_3-LNCaP cells were allowed to attach to either OPN or VN. To examine PI 3-kinase activation, we analyzed the levels of Ser^{473} phosphorylation of AKT, a downstream effector of PI 3-kinase, as a sensitive readout of PI 3-kinase activity (56). PC3 cells stimu-

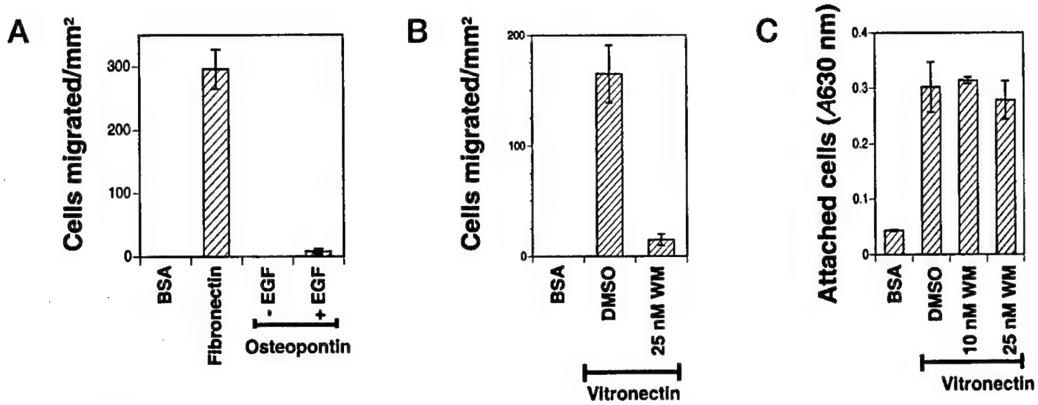


FIG. 7. **β_3-LNCaP transfectants do not migrate on OPN but migrate on VN via PI 3-kinase activation.** *A*, migration of β_3-LNCaP cells at 37 °C for 4 h in the Transwell chambers coated on both sides with BSA (10 mg/ml), FN (3 μg/ml), and OPN (10 μg/ml) is shown. OPN was tested in the presence or absence of 200 ng/ml EGF. Migration on FN is shown as a control. *B*, migration of β_3-LNCaP cells on VN (3 μg/ml) in the presence of 25 nM wortmannin (*WM*) at 37 °C for 4 h is shown. Me_2SO (*DMSO*) was used as a vehicle for wortmannin. *C*, adhesion of β_3-LNCaP cells to VN (3 μg/ml) performed in the presence of 10 and 25 nM wortmannin at 37 °C for 2 h is shown. In *panels A–C*, BSA (10 mg/ml) was used as negative control. Attached cells were fixed in 3% paraformaldehyde at 4 °C for 30 min, stained with 0.5% crystal violet at room temperature for at least 2 h, and described under "Experimental Procedures." Comparable levels of adhesion were observed at the used concentrations of each substrate. Triplicate observations were performed. All experiments were repeated at least three times with consistent results. *A–C*, *error bars*, mean ± S.E. (n = 3).

lated the PI 3-kinase/AKT signaling pathway on OPN more significantly than on VN through $\alpha_v\beta_3$ or than on FN through β_1 integrins (Fig. 8*A*), although they attached to OPN, VN, and FN equally well (Fig. 8*B*). The maximum levels of AKT phosphorylation on OPN were observed between 30 and 45 min. As shown in Fig. 9*A*, β_3-LNCaP cell adhesion to OPN did not induce AKT Ser^{473} phosphorylation, whereas adhesion to VN induced a significant increase in AKT Ser^{473} phosphorylation. Densitometric analysis performed using three separate exposures in a linear range showed a 13- to 18-fold increase in AKT Ser^{473} phosphorylation on VN and a 1- to 2-fold increase on OPN. AKT phosphorylation induced by EGF was detected at 20, 30, 60, and 120 min on cells attached to their extracellular matrix (not shown); however EGF did not induce AKT Ser^{473} phosphorylation in β_3-LNCaP cells attached to OPN (Fig. 9*A*) nor did it increase AKT phosphorylation when these cells attached to VN (Fig. 9*B*).

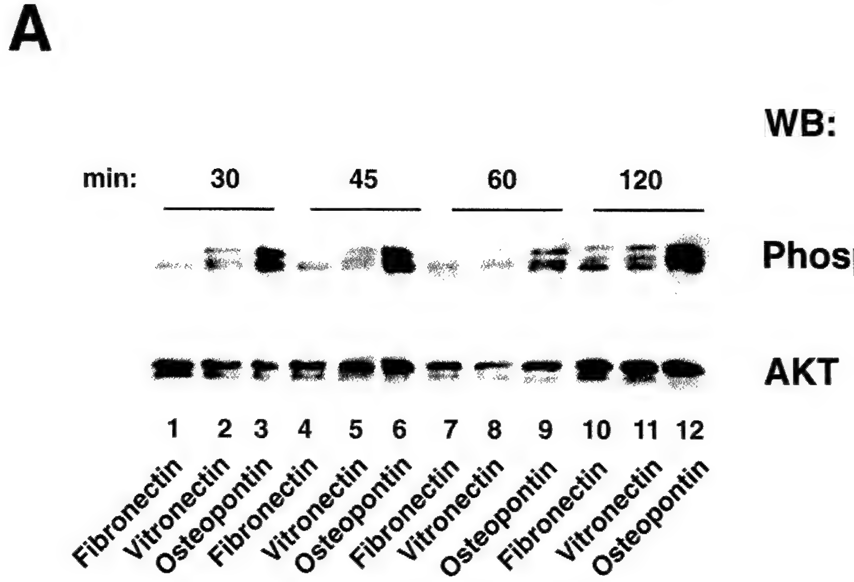
A pharmacologic approach was also used to investigate the role of other downstream effectors of FAK that have been previously shown to mediate cell migration, ERK1 and -2 (60, 61). An inhibitor of MEK-1 in the MAP kinase pathway, PD98059 (69), was tested to analyze its effect on β_3-LNCaP and PC3 cell migration. PD98059 had no effect on migration of either cell type on VN, although it did inhibit endothelial cell migration (data not shown and Ref. 61). Activation of ERK1/2 occurred in PC3 cells attached to both VN and OPN substrates (Fig. 10*A*); in contrast, β_3-LNCaP cells attached to VN and OPN did not activate ERK1/2 phosphorylation at any of the time points analyzed (Fig. 10*B* and not shown); ERK1/2 activation was detected in response to EGF treatment in cells attached to their matrix in tissue culture dishes (Fig. 10*B*). In Fig. 10*A*, ERK1/2 activation is longer than other cells tested in our laboratory in similar conditions; these results do not have a mechanistic explanation at this time. However, the observed sustained activation seems to be non-integrin-dependent because it is seen in cells in suspension as well after 30 min. Thus, ERK1/2 activation did not play a role or correlate with either PC3 or β_3-LNCaP cell migration.

Our previous report showed that FAK played a predominant role in mediating cell migration on VN, because migration was inhibited by expression of FAK-related non-kinase (5). It has been shown that PI 3-kinase forms a complex with FAK, acts as a downstream effector of FAK, and controls cell migration (53). In β_3-LNCaP cells, FAK is phosphorylated in response to $\alpha_v\beta_3$ engagement by OPN and by VN (Fig. 11). Densitometric analysis performed using three separate exposures in a linear range showed the following increase in FAK phosphorylation: 8.5-fold increase on OPN and 9.4-fold on VN in presence of EGF and 8.1-fold on VN in the absence of EGF. EGF stimulation did induce comparable levels of HER tyrosine phosphorylation in cells in suspension or attached to VN or OPN (data not shown), thus indicating that HER is functional in all tested conditions; however, EGF did not increase FAK-tyrosine phosphorylation either on VN or in suspended cells (Fig. 11). In conclusion, on OPN substrates, $\alpha_v\beta_3$-mediated signaling events fail to be activated downstream of FAK at the level of PI 3-kinase/AKT activation.

DISCUSSION

This study shows that $\alpha_v\beta_3$ is expressed in multiple functional states and that its ability to mediate cell migration and intracellular signaling pathways is substrate-specific and functional state-dependent. In PC3 cells, $\alpha_v\beta_3$ mediates cell adhesion, migration, and PI 3-kinase/AKT pathway activation on VN and OPN. In contrast, adhesion to OPN of noninvasive LNCaP cells upon exogenous expression of $\alpha_v\beta_3$ requires its activation by EGF although $\alpha_v\beta_3$ is in a functional state that allows adhesion to a different ligand, VN, in the absence of EGF. Furthermore, in LNCaP cells, while $\alpha_v\beta_3$ mediates cell migration and PI 3-kinase/AKT pathway activation on VN, adhesion to OPN fails to support cell migration and PI 3-kinase/AKT pathway activation mediated by $\alpha_v\beta_3$. This is the first report that shows an integrin ligand-mediated phenotypical alteration that reverts a migratory cell into a nonmigratory cell via engagement of the same integrin. We conclude that $\alpha_v\beta_3$ exists in multiple functional states that can bind either VN selectively or both VN and OPN and that can differentially activate cell migration and the PI 3-kinase/AKT signaling pathway in a ligand-specific manner. The results highlight a versatile role for $\alpha_v\beta_3$ in the regulation of the PI 3-kinase/AKT pathway and in a substrate-dependent control of cell invasion.

We show for the first time that EGF reverts a form of $\alpha_v\beta_3$ that does not recognize OPN in an OPN-binding form. Although the mechanism of activation remains to be identified, EGF effect is not due to a change in integrin expression on the cell surface because EGF regulates cell adhesion to OPN with-



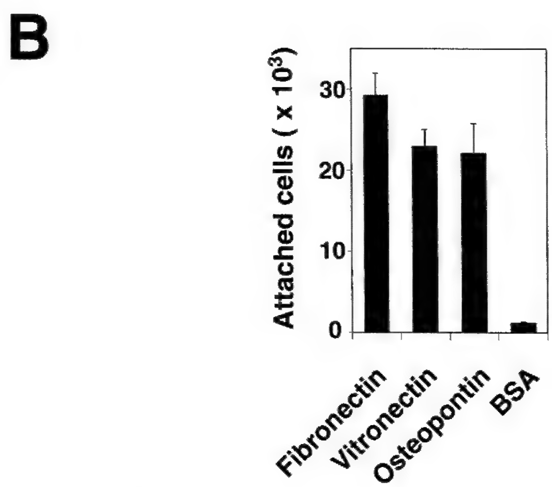


FIG. 8. **Increased AKT Ser[473] phosphorylation in response to $\alpha_v\beta_3$ engagement by OPN in PC3 cells.** *A*, PC3 cells starved in serum-free medium for 24 h were seeded on FN (2 μg/ml)-, VN (0.3 μg/ml)-, and OPN (0.3 μg/ml)-coated Petri dishes at 37 °C for the indicated times. The attached cells were lysed on the dish as described under "Experimental Procedures." Thirty μg of cell lysate per lane were loaded on 10% SDS-PAGE. Phosphorylation of AKT is shown by immunoblotting (*WB*) using a polyclonal antibody to phospho-AKT Ser[473] (0.05 μg/ml, *top panel*). Protein loading control is shown on the *lower panel* by AKT immunoblotting with polyclonal antibody to AKT (0.1 μg/ml). *B*, adhesion of ^{51}Cr-labeled PC3 cells at 37 °C for 2 h on 96-well plates coated with OPN (0.3 μg/ml), VN (0.3 μg/ml), or FN (2 μg/ml) is shown. Cell adhesion to BSA (10 mg/ml)-coated wells is shown as a negative control. *Error bars*, mean ± S.E. (n = 3).

out a significant change in integrin expression (Fig. 1). EGF-downstream players that might potentially activate $\alpha_v\beta_3$ are protein kinase C, known to be involved in mediating $\alpha_v\beta_3$ activation (38), and HER through its direct association with integrins (70); however, additional modulators, such as integrin-associated proteins (71, 72), might be responsible for changes in ligand binding or post-ligand binding activities. Similar to our findings, activation-independent (fibrinogen) and -dependent (prothrombin) ligands for $\alpha_v\beta_3$ have been shown by Byzova and Plow (38) suggesting that a sophisticated mechanism of tight regulation and ligand selection involves $\alpha_v\beta_3$.

The EGF receptor has been shown to synergize with $\alpha_v\beta_5$ to increase cell migration (73); LNCaP cells express low levels of β_5 and large amounts of β_1 (Fig. 1 and Ref. 5). However, these previously described OPN receptors did not play a role in the adhesion of these cells to OPN in our experimental system, since first, parental or mock-LNCaP cells that did not express $\alpha_v\beta_3$ did not adhere to OPN in response to EGF stimulation and second, an antibody to $\alpha_v\beta_5$ did not inhibit OPN adhesion of β_3-LNCaP cells. We conclude that EGF and its receptor HER synergize with $\alpha_v\beta_3$ in a substrate-specific manner on OPN but not on VN. This change required for β_3-LNCaP cell adhesion to OPN did not support cell migration on OPN although these cells migrated on VN. The ability of $\alpha_v\beta_3$ to mediate cell migration is therefore substrate-specific. Invasive PC3 cells have the ability to up-regulate cell migration through $\alpha_v\beta_3$ on OPN; therefore, it is conceivable that when cancer cells lose the ability to select their cell binding partners by uncoupling/de-regulating the synergistic activity of $\alpha_v\beta_3$ integrin and HER,

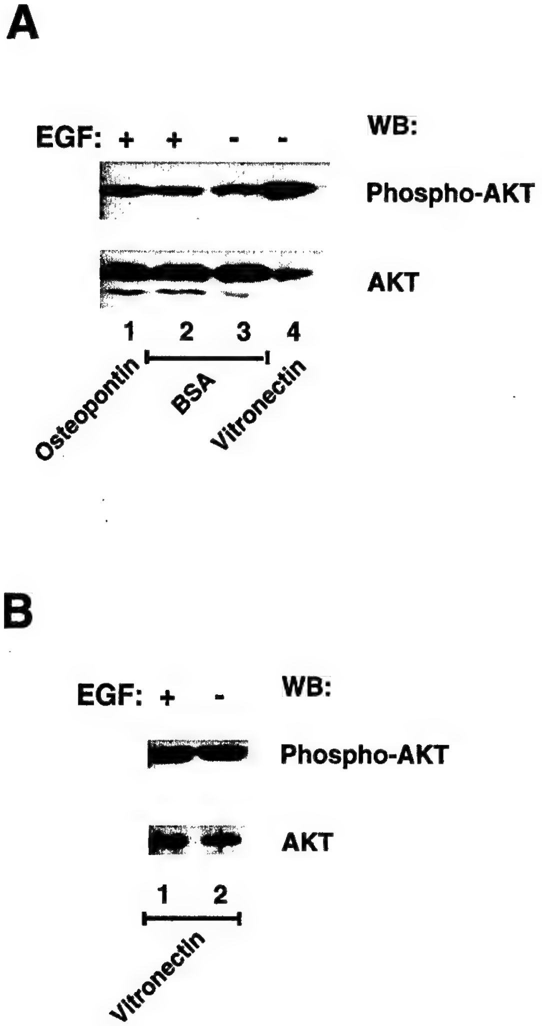


FIG. 9. **Adhesion of β_3-LNCaP transfectants to OPN does not stimulate AKT Ser^{473} phosphorylation.** *A*, *top panel*, AKT phosphorylation of β_3-LNCaP transfectants attached to OPN (10 μg/ml) or VN (3 μg/ml) or held in suspension (10 mg/ml BSA) at 37 °C for 3 h was measured by immunoblotting using a polyclonal antibody to phospho-AKT Ser^{473} (0.05 μg/ml). Protein loading control is shown on the *bottom panel* by AKT immunoblotting (*WB*) with polyclonal antibody to AKT (0.1 μg/ml). *B* shows phospho-AKT Ser^{473} (*top panel*) and AKT protein loading (*bottom panel*) of β_3-LNCaP transfectants attached to VN (3 μg/ml) at 37 °C for 3 h with and without EGF. The experiments were repeated at least three times with consistent results.

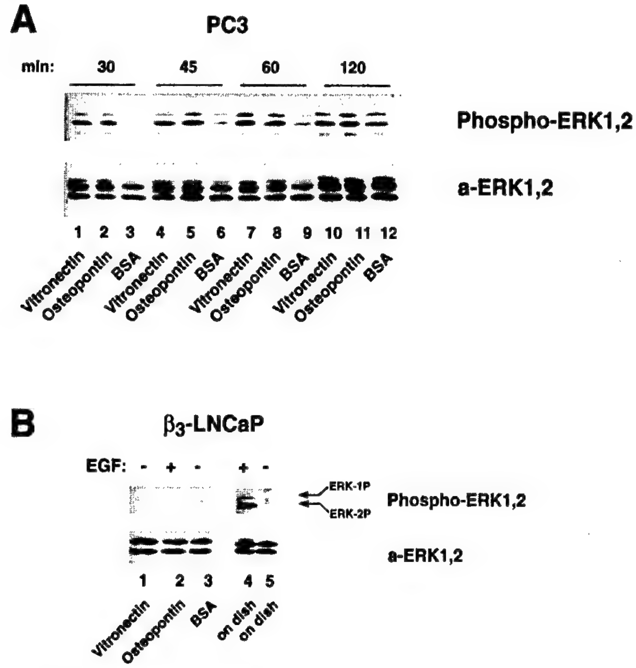


FIG. 10. **Activation of ERK1/2 and FAK signaling pathways on OPN and VN.** *A*, ERK1/2 phosphorylation was tested using a monoclonal antibody to phospho-ERK1/2 (*top panel*), and protein loading was confirmed (*bottom panel*) using a polyclonal antibody to ERK1/2. The experiments were repeated at least twice with consistent results. *B*, cell lysates from cells attached to OPN (10 μg/ml) or VN (3 μg/ml) or to their matrix in tissue culture dishes (on dish) in the absence of EGF or in the presence of EGF (200 ng/ml) were analyzed using 0.1 μg/ml rabbit affinity-purified antibody to ERK1/2 (*bottom panel*) and a monoclonal antibody to phospho-ERK1/2 (*top panel*). The experiments were repeated at least two times with consistent results.

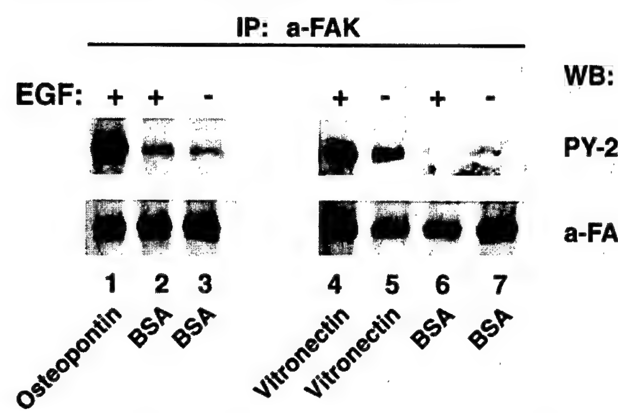


FIG. 11. **Tyrosine phosphorylation of FAK in β_3-LNCaP cells attached to OPN or VN.** FAK tyrosine phosphorylation of β_3-LNCaP cell transfectants was measured using PY20 (1 μg/ml) immunoblotting (*top panels*) of immunocomplexes precipitated by C-20 polyclonal antibody to FAK (0.5 μg) from β_3-LNCaP transfectant detergent lysates. Lysates were prepared from cells attached to OPN (10 μg/ml)- or VN (3 μg/ml)-coated dishes in the presence or absence of EGF (200 ng/ml). The same membrane was stripped, and FAK protein levels were analyzed using 0.1 μg/ml affinity-purified antibody to FAK (*bottom panels*). FAK tyrosine phosphorylation is expressed as -fold increase over the levels detected in cells held in suspension. The experiments were repeated at least three times with consistent results.

such as in PC3 cells, they migrate in response to engagement by multiple $\alpha_v\beta_3$ ligands.

Among the three known pathways that mediate cell migration and are activated by integrins: FAK, PI 3-kinase/AKT, and MAP kinase pathways, we have shown that the FAK (5) and the PI 3-kinase/AKT pathways support migration on VN in β_3-LNCaP cells and on VN and OPN in PC3 cells. The MAP kinase pathway did not play a role in either β_3-LNCaP or PC3 cell migration because PD98059 did not block cell migration (not shown). It should be pointed out that AKT has the ability to support cell migration mediated by vascular endothelial growth factor in endothelial cells (57); it is not known, however, whether this mechanism is active in other cells. We show that $\alpha_v\beta_3$-OPN interaction mediates FAK tyrosine phosphorylation but this signal, although necessary, is not sufficient to mediate cell migration in noninvasive cells. PI 3-kinase, a mediator of integrin and growth factor activities including EGF (74), is known to act as a downstream effector of FAK and to control cell migration activated by cell adhesion to extracellular matrix proteins (53, 54). Integrin-mediated adhesion to the extracellular matrix proteins stimulates the association of the p85 regulatory PI 3-kinase subunit with FAK through FAK Tyr^{397} (51, 52); FAK binding to PI 3-kinase has been demonstrated to activate the latter one (53). Because FAK is tyrosine-phosphorylated in response to OPN adhesion mediated by EGF, we conclude that a block at

the level of PI 3-kinase/AKT activation downstream of FAK explains the failure of β_3-LNCaP cells to migrate, although $\alpha_v\beta_3$ and the PI 3-kinase/AKT pathway are fully functional in these cells upon $\alpha_v\beta_3$ engagement by VN. The data suggest that the generated β_3-LNCaP cells are a model system that allows the study of the $\alpha_v\beta_3$ effectors that mediate cell migration downstream of FAK. It remains to be analyzed whether FRNK, a negative regulator of FAK that we have shown inhibits VN-mediated migration in β_3-LNCaP cells (5), specifically inhibits FAK/PI 3-kinase interaction. It should be stressed that the PI 3-kinase/AKT pathway might also control cell adhesion, as shown by Byzova and Plow since in this study (43) wortmannin did inhibit both cell adhesion and migration after a 30-min preincubation; however, we did not observe wortmannin inhibition of cell adhesion to VN and OPN in our system due to either a cell type-specific effect or to the lack of preincubation with wortmannin in our experimental system. PTEN, a lipid phosphatase that prevents FAK and PI 3-kinase/AKT pathway activation (75, 76) and down-regulates cell motility and directionality (77) is not expected to contribute to the migration of these cells, because LNCaP and PC3 cells have been shown to express a mutated and a deleted PTEN, respectively (78). In both cell types, PI 3-kinase/AKT pathway activation is controlled by integrins in the absence of an active PTEN, confirming that other molecules such as either Cdc42 (58) or ILK (59) could control integrin-mediated cell migration.

We show here that in β_3-LNCaP cells a differential activation of the PI 3-kinase/AKT pathway by $\alpha_v\beta_3$ occurs: OPN interaction with $\alpha_v\beta_3$ does not activate the PI 3-kinase/AKT pathway, whereas VN does. It should be noted that PI 3-kinase/AKT pathway is stimulated via OPN engagement of $\alpha_v\beta_3$ in PC3 cells (Fig. 8*A*) and in osteoclasts (79), thus indicating that the specific failure to activate the PI 3-kinase pathway is cell type-dependent. Because OPN-null mice generate significantly smaller metastases than wild type mice (21), it is thus conceivable that in the event LNCaP or another noninvasive cell will migrate to a metastatic site where OPN is predominantly expressed, the interaction of $\alpha_v\beta_3$ with OPN will not provide a migratory or, alternatively, survival signal for these cells. Recently, the role of AKT in promoting cell survival of androgen-sensitive LNCaP cells but not of androgen-insensitive PC3 cells has been shown (80). It is noted that because AKT activation promotes cell survival (48) and LNCaP cells undergo apoptosis in the presence of the PI 3-kinase inhibitor, wortmannin (80), the failure of OPN to stimulate AKT activation might result in apoptosis of these poorly tumorigenic cells. It remains to be determined whether direct $\alpha_v\beta_3$ integrin engagement in prostate cells prevents apoptosis by activation of the PI 3-kinase/AKT pathway. The synergistic activity of $\alpha_v\beta_3$, OPN, and the downstream PI 3-kinase/AKT pathway might balance proliferative, apoptotic, and migratory stimuli, thus playing a crucial role in tumor growth and metastatic events *in vivo*.

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Regulation of mRNA and Protein Levels of β1 Integrin Variants in Human Prostate Carcinoma

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Alterations of integrin expression levels in cancer cells correlate with changes in invasiveness, tumor progression, and metastatic potential. The β1C integrin, an alternatively spliced form of the human β1 integrin, has been shown to inhibit prostate cell proliferation. Furthermore, β1C protein levels were found to be abundant in normal prostate glandular epithelium and down-regulated in prostatic adenocarcinoma. To gain further insights into the molecular mechanisms underlying abnormal cancer cell proliferation, we have studied β1C and β1 integrin expression at both mRNA and protein levels by Northern and immunoblotting analysis using freshly isolated neoplastic and normal human prostate tissue specimens. Steady-state mRNA levels were evaluated in 38 specimens: 33 prostatic adenocarcinomas exhibiting different Gleason's grade and five normal tissue specimens that did not show any histological manifestation of benign prostatic hypertrophy. Our results demonstrate that β1C mRNA is expressed in normal prostate and is significantly down-regulated in neoplastic prostate specimens. In addition, using a probe that hybridizes with all β1 variants, mRNA levels of β1 are found reduced in neoplastic *versus* normal prostate tissues. We demonstrate that β1C mRNA down-regulation does not correlate with either tumor grade or differentiation according to Gleason's grade and TNM system evaluation, and that β1C mRNA levels are not affected by hormonal therapy. In parallel, β1C protein levels were analyzed. As expected, β1C is found to be expressed in normal prostate and dramatically reduced in neoplastic prostate tissues; in contrast, using an antibody to β1 that recognizes all β1 variants, the levels of β1 are comparable in normal and neoplastic prostate, thus indicating a selective down-regulation of the β1C protein in prostate carcinoma. These results demonstrate for the first time that β1C and β1 mRNA expression is down-regulated in prostate carcinoma, whereas only β1C protein levels are reduced. Our data highlight a selective pressure to reduce the expression levels of β1C, a very efficient inhibitor of cell proliferation, in prostate malignant transformation. ***(Am J Pathol 2000, 157:1727–1734)***

Prostatic carcinoma is the most common type of cancer found in men in the western world and its distant metastases become a life-threatening event in tumor bearing patients. Although the biology of prostate cancer and metastases is poorly understood, it is becoming clear that major determinants of the malignant or hyperplastic phenotype are adhesion molecules, various growth factors, and their receptors, whose inappropriate expression or loss disrupts normal regulation of cell proliferation and differentiation.[1]

Integrins are a superfamily of cell surface adhesion receptors that play a critical role in tumor progression and metastases as well as in a number of physiological processes such as inflammation, cell adhesion, migration, proliferation, survival, and differentiation.[2–5] Integrins are receptors for extracellular matrix proteins such as fibronectin, vitronectin, collagen, and laminin.[6] In addition to mediate cell adhesion to the extracellular matrix, integrins also transduce biochemical signals into the cell thus regulating cell proliferation and differentiation.[7,8]

Integrins are transmembrane glycoproteins composed of α and β subunits that associate to form a heterodimer; 16 α subunits and eight β subunits, that associate to form at least 22 different receptors, have been discovered to date.[7,8] Each subunit has a large extracellular domain, a single transmembrane domain and a short cytoplasmic domain.[9] The role of the integrin cytoplasmic domain in modulating integrin functions and signaling events is well established.[10,11]

Alternatively spliced variants of the integrin cytoplasmic domain have been described for some of the α and

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Table 1. Clinicopathological Features of 33 Patients with Prostate Carcinoma

Patients	Age (years)	Gleason's grade	Stage	Hormonal therapy (months)
N1	60	Control		
N2	62	Control		
N3	69	Control		
N4	72	Control		
N5	65	Control		
K1	57	≥7	T3N0M0	–
K2	63	≥7	T3N1M0	–
K3	77	≥7	T2N0M0	–
K4	70	<7	T2N0M0	3
K5	67	≥7	T2N0M0	–
K6	63	≥7	T3N0M0	–
K7	70	≥7	T3N0M0	6
K8	59	≥7	T3N1M0	–
K9	66	≥7	T3N1M0	–
K10	57	≥7	T3N1M0	–
K11	67	≥7	T2N0M0	6
K12	67	≥7	T3N0M0	–
K13	76	<7	T2N0M0	3
K14	76	≥7	T3N0M0	1
K15	59	≥7	T3N0M0	–
K16	61	≥7	T3N0M0	–
K17	64	<7	T2N0M0	–
K18	69	≥7	T2N0M0	6
K19	72	≥7	T3N1M0	–
K20	64	<7	T3N0M0	–
K21	59	≥7	T3N0M0	–
K22	60	≥7	T2N0M0	6
K23	69	≥7	T2N0M0	–
K24	63	≥7	T3N1M0	–
K25	62	≥7	T4N1M0	–
K26	66	≥7	T2N0M0	6
K27	76	≥7	T3N0M0	–
K28	70	≥7	T3N0M0	–
K29	66	≥7	T3N0M0	–
K30	75	≥7	T3N0M0	–
K31	67	≥7	T4N0M0	–
K32	67	<7	T2N0M0	3
K33	68	≥7	T3N0M0	–

K, prostate carcinoma; N, normal prostate; –, no hormonal therapy.

β subunits.[11] Alternative splicing events between exon 6 and exon 7 of the β1 integrin subunit generate four different isoforms.[11] A β1 isoform, β1C, was found to be expressed in normal prostate epithelial cells.[11,12] Its cytoplasmic domain consists of 26 amino acids encoded by exon 6, and 48 amino acids derived from an additional exon, exon C and part of exon 7, in the β1 integrin gene. It has been demonstrated that β1C expression inhibits cell proliferation and causes growth arrest in the late G_1 phase of the cell cycle.[13–15] Recent studies have also demonstrated that β1C causes up-regulation of the cyclin kinase inhibitor $p27^{Kip1}$ protein levels in prostate cells shedding new lights into the molecular mechanisms underlying prostate cancer progression.[16]

Although several groups have analyzed integrin expression in prostate cancer *in vitro* or *in vivo*[17–20] at the protein levels, very few studies have described integrin mRNA expression in prostate malignant transformation either *in vitro* or *in vivo*.[21,22] We have studied β1C and β1 expression at both mRNA and protein levels by Northern and immunoblotting analysis using specimens from 33 patients affected by prostatic carcinoma. Our results demonstrate for the first time that β1C and β1 mRNA expression is down-regulated in prostate carcinoma, whereas only β1C protein levels are reduced.

Materials and Methods

Tissue Specimens

This study was performed using 38 prostate specimens (Table 1) obtained from either patients who underwent radical cystoprostatectomy for bladder carcinoma noninvolving the prostate (five specimens) or patients with prostate cancer (33 specimens) hospitalized at the Department of Urology of the University of Bari, School of Medicine, in the years 1998 to 1999. Informed consent was obtained from all patients. Soon after surgical removal of the prostate, a sample was taken from all specimens, snap-frozen, and cryopreserved in liquid nitrogen for RNA extraction and immunoblotting analysis. The remaining tissue samples were fixed in 10% neutral-buffered formalin for 12 to 24 hours, embedded in paraffin, and stained with hematoxylin and eosin (H&E). H&E-stained sections were reviewed, and the tumor grade,

according to Gleason's criteria[23] and the stage of tumor, according to TNM system,[24] were estimated in each tumor sample.

A sample of normal human liver, obtained during cholecystectomy, was also used to generate mRNA.

RNA Extraction and Northern Blot Analysis

Frozen tissue samples were pulverized to a fine powder and cellular RNA was extracted using the guanidinium isothiocyanate-cesium chloride procedure.[25] Total RNA (25 μg) isolated from the tissues was electrophoresed through 1% denaturing agarose gel containing 660 mmol/L formaldehyde, and transferred[26] to a nylon membrane (Hybond N^+; Amersham, Milan, Italy). The filters were subsequently prehybridized overnight at 42°C with a buffer consisting of 50% formamide, 5× Denhardt's solution (1% Ficoll 400, 1% polyvinylpyrrolidone, 1% bovine serum albumin), 5× sodium chloride/sodium phosphate/ethylenediaminetetraacetic acid (SSPE) (3 mol/L NaCl, 200 mmol/L $Na_2H_2PO_4$, pH 7.0, 19 mmol/L ethylenediaminetetraacetic acid), 0.5% sodium dodecyl sulfate (SDS) and 100 μg/ml of sonicated salmon sperm DNA. The filters were then hybridized for 20 hours at 42°C by adding 3×10^6 cpm of ^{32}P-labeled probe/ml to the prehybridization solution. The filters were washed once with 2× SSPE, 0.1% SDS for 10 minutes at room temperature, then with 1× SSPE, 0.1% SDS at 42°C, followed by several washes in 0.1× SSPE, 0.1% SDS, at 65°C and finally exposed at −80°C overnight or longer to Kodak X-Omat AR 5 film (Kodak, Rochester, NY). Radiolabeled probes were generated using the Megaprime DNA labeling kit (Amersham), 5 μl of α-^{32}P-dCTP (3,000 Ci/mmol, Amersham)[27] and 25 ng of double-stranded either 116-bp fragment specific for the β1C integrin or a full-length human β1 cDNA.[12] The specific 116-bp β1C fragment (nucleotides 2435 to 2550)[12] was generated by polymerase chain reaction using pBluescript β1C plasmid as template and the resulting fragment was subcloned in the pBluescript vector. mRNA levels were normalized using ribosomal 28S RNA, a constitutively expressed gene.[28] For this purpose, blots were stripped in 0.1% boiling SDS and reprobed with the radiolabeled ^{32}P-28S cDNA probe. Quantitative analysis was performed by densitometric scanning of the autoradiographs using a Bio-Rad GS-700 densitometer (Bio-Rad, Richmond, CA); multiple exposures of the same Northern blots in a linear range were performed. The ratio between the 4.2-kb long β1C mRNA levels and the 28S rRNA levels was calculated for each sample to take into account differences in RNA loading. The average of either β1C or β1 mRNA expression levels in control normal prostate derived from five patients was set at 100 (arbitrary units). β1C or β1 mRNA levels in neoplastic prostate were calculated as percentage of normal prostate mRNA levels hybridized on the same filter. For each specimen, the mean value (±SEM) of results obtained in at least three experiments was calculated.

Immunoblotting

Either normal or tumor frozen tissue specimens obtained from radical prostatectomy were homogenized in lysis buffer containing 0.1% SDS, 1% Nonidet P-40, 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 200 mmol/L LiCl, 5 mmol/L ethylenediaminetetraacetic, 10% glycerol, 10 μg/ml aprotinin, 120 μg/ml leupeptin, 170 μg/ml phenylmethylsulfonyl fluoride. The homogenate was sonicated for 20 seconds, then centrifuged for 30 minutes at 14,000 × *g* at 4°C. Two-mercaptoethanol (1%) was added to each lysate for 30 minutes at 4°C to further solubilize potentially cross-linked molecules and 150 μg of tissue extracts were electrophoresed on 7.5% SDS-polyacrylamide gel electrophoresis under reducing conditions. Immunoblotting was performed as previously described[20] using either 5 μg/ml rabbit polyclonal affinity-purified antibody to β1C integrin or 1 μg/ml mouse monoclonal antibody to β1 integrin (Transduction Laboratories, Milan, Italy) or 10 μg/ml of antibody to β-tubulin (Sigma, St. Louis, MO) for 16 hours at 4°C in Tris-buffered saline/Tween 20 (TBS-T) (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.2% Tween-20). The membrane was then washed three times in TBS-T and incubated with horseradish-peroxidase-conjugated goat affinity-purified antibody to either rabbit or mouse IgG (Amersham), in TBS-T for 1 hour at room temperature. After three washes in TBS-T, the proteins were visualized using the Amersham enhanced chemiluminescent system according to the manufacturer's instructions. Densitometric values for immunoreactive bands were quantified using a GS-700 Imaging Densitometer (Bio-Rad). β1C and β1 protein levels were calculated as percentage of control (normal prostate tissue) upon normalization using β-tubulin as control for protein loading.

HL60 Cells

Human leukemia HL60 cells were grown in RPMI 1640 (Gibco, Life Technologies, Milan, Italy), with 50 μg/ml gentamicin, 2 mmol/L glutamine, and 15% inactivated fetal calf serum, at 37°C in presence of 5% CO_2. Total RNA from differentiated cells was prepared 24 hours after incubation with 160 nmol/L TPA (or PMA phorbol-12-myristate-13-acetate; Sigma) as previously described.[29]

Statistical Analysis

Data are reported as the mean ± SEM for the indicated experiments. Statistical analysis was performed using the Student's *t*-test. All experiments were repeated at least three times.

Results

β1C mRNA Expression in Neoplastic and Normal Prostate Tissues

This study was performed using 38 prostate specimens obtained from patients with either prostate or bladder

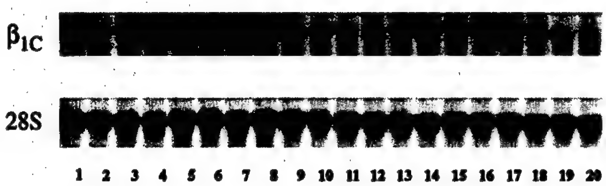

Figure 1. β1C mRNA expression is down-regulated in human prostate neoplastic tissues. Total RNA was isolated from five normal and 13 neoplastic prostate specimens. β1C mRNA expression was evaluated by Northern blotting using the 116-bp β1C-specific probe shown in Figure 2. Twenty-five μg total RNA were used for each sample. **Lane 1:** RNA from HL60 cells was used as positive control. **Lane 2:** RNA from human liver was used as negative control. **Lanes 3** to **7:** RNA from normal prostate tissues. **Lanes 8** to **20:** RNA from neoplastic prostate tissues. To normalize the amount of total RNA loaded for each sample, the blot was stripped and rehybridized using a 28S rRNA probe.

cancer. The patients were divided into two groups (Table 1). The first group included five patients (N1 to N5; age range, 60 to 72 years) with normal prostate who underwent radical cystoprostatectomy for bladder carcinoma noninvolving the prostate. The second group included 33 patients (K1 to K33; age range, 57 to 77 years) with histologically proven prostatic adenocarcinoma, who underwent radical prostatectomy. In the first group, normal prostatic tissue was histologically confirmed. In the second group, the grade of tumor differentiation and the stage of tumor were estimated in each tumor sample according to Gleason's criteria and to TNM system, respectively. Based on Gleason's criteria[23] the prostate specimens were divided in five moderately differentiated (Gleason's score <7) and 28 poorly differentiated (Gleason's score ≥7) prostate tumors; based on TNM system,[24] the specimens were divided in 11 T2N0M0 (stage II), 14 T3N0 M0 (stage III), and six T3N1M0, one T4N0M0, and one T4N1M0 (stage IV) prostate tumors. A neoadjuvant hormonal therapy consisting of the association of luteinizing hormone releasing hormone analogue (goserelin depot, 3.6 mg/q 28 days) and a nonsteroidal anti-androgen (bicalutamide, 50 mg/day) was administered to nine patients affected by prostate carcinoma (Table 1) before surgery to reduce prostate and tumor volume and to obtain downstaging of the tumor, as reported in preliminary clinical trials.[30,31]

Expression of β1C and β1 was examined at the RNA level in 38 prostate tissues using either a β1C-specific probe (Figure 2) or a β1 full-length probe that hybridizes with all of the β1 variants.[11] Steady-state levels of β1C (Figure 1 and not shown) and β1 (Figure 3 and not shown) mRNA were evaluated by Northern blotting analysis of total RNA isolated from 33 neoplastic and five normal tissues (Table 1). Because of the low amount of RNA obtained from the tissue samples, total RNA rather than poly(A+) RNA was analyzed. A 4.2-kb transcript was detected in all samples (Figure 1). This band corresponds to the β1C mRNA because the probe used is specific for exon C, which is found only in β1C (Figure 2). Total RNA extracted from TPA-differentiated HL60 cells and from human liver, was used as positive and negative controls for β1C mRNA expression, respectively (Figure 1, lanes 1 and 2), because β1C is expressed in TPA-differentiated HL60 cells and is barely detectable in normal human liver.[12,15] As expected, low levels of β1C

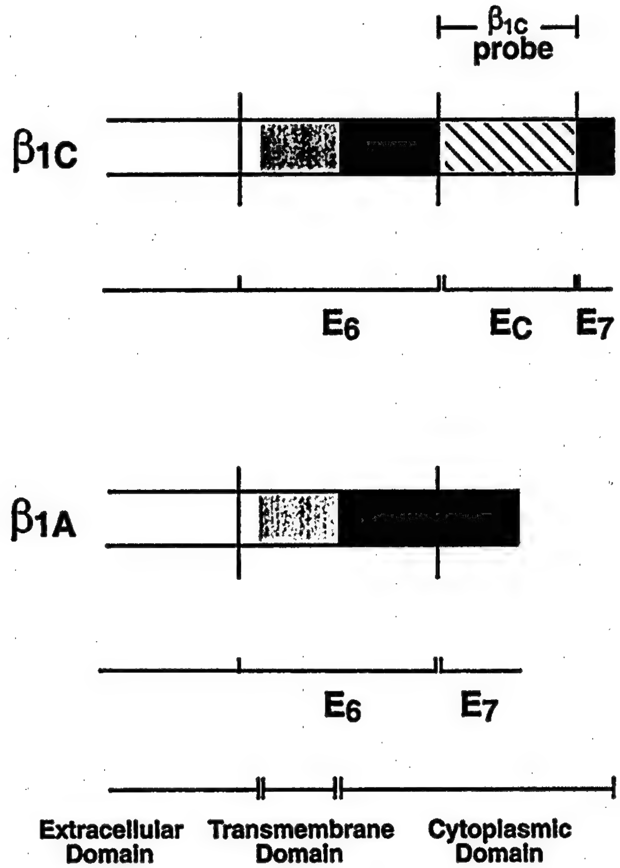

Figure 2. β1C-specific probe. Schematic drawing showing β1C and β1A cytoplasmic domains.[11] The specific 116-bp β1C probe is shown. The 116-bp fragment used in Northern blotting analysis (see Figure 1) was generated by polymerase chain reaction using pBluescript-β1C plasmid as template. E, exon.

mRNA were found in normal liver compared with HL60 cells (Figure 1, lanes 1 and 2). Northern blotting analysis showed a significant decrease (49 ± 4% decrease) of β1C mRNA levels in neoplastic tissues (Figure 1, lanes 8 to 20) compared with normal prostate tissues (Figure 1, lanes 3 to 7). Decreased β1C steady-state mRNA levels were detected in 94% of the prostatic carcinoma specimens compared with normal prostate samples and the differences were statistically significant ($P < 0.005$; Figure 4). In one instance (K16), β1C mRNA expression was found increased (121 ± 8%) compared with the levels in normal prostate tissues, whereas in a different specimen (K10), β1C mRNA levels were comparable (100 ± 4%) to normal prostate levels (Figure 4).

Using full-length β1 cDNA, that hybridizes with all β1 variants,[14] as probe, we analyzed the 38 prostate tissue specimens described above. The results show that among the 33 prostatic adenocarcinoma, 29 showed reduced β1 mRNA levels when compared with the five normal prostate tissues (Figure 3 and not shown).

The results show that β1C as well as β1 mRNA levels are reduced in prostatic adenocarcinoma compared with normal prostate tissues.

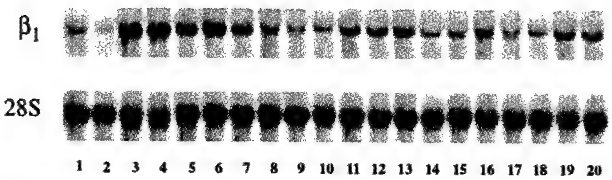

Figure 3. β1 mRNA expression is down-regulated in human neoplastic prostate tissues. Total RNA was isolated from five normal and 13 neoplastic prostate specimens. β1 mRNA levels were evaluated by Northern blotting using a human full-length β1 probe. **Lane 1:** RNA from HL60 cells. **Lane 2:** RNA from human liver. **Lanes 3** to **7:** RNA from normal prostate tissues. **Lanes 8** to **20:** RNA from neoplastic prostate tissues. To normalize the amount of total RNA loaded for each sample, the blot was stripped and rehybridized using a 28S rRNA probe.

β1C mRNA Expression and Clinical Progression

To investigate whether β1C expression is associated with tumor stage, correlation of β1C mRNA levels with clinicopathological parameters (Gleason's grade and tumor stage) was evaluated. As shown in Figure 5A, β1C mRNA expression was comparable in patients with Gleason's grade <7 (57 ± 7%, $n = 5$) and Gleason's grade ≥7 (50 ± 5%, $n = 28$). In parallel, the specimens were analyzed using the TNM system for tumor stage classification (Figure 5B). The differences in mRNA levels in specimens at different stages were not statistically significant ($P > 0.05$); in fact, as shown in Figure 5B, β1C mRNA expression was comparable, although lower than normal prostate controls, in stage II, III, and IV tumors (48 ± 6%, 52 ± 8%, and 56 ± 7%, respectively). Moreover, we investigated whether a correlation between β1C mRNA expression and hormonal therapy occurred. As shown in Figure 6, the differences between patients with 3- or 6-month hormonal treatment and patients who did not undergo hormonal therapy were not significant ($P >$ 0.05 in both cases) although they were reduced *versus* normal prostate controls (64 ± 10%, 38 ± 7%, 52 ± 5%, respectively). In the only available case where 1-month hormonal therapy had been administered, there was a statistically significant increase (156 ± 4%) with respect to the patients that had not received any therapy; however, the results related to short-term (1 month) therapy need to be further investigated using a larger number of cases, when available.

In conclusion, down-regulation of β1C mRNA levels did not correlate with either tumor grade, or tumor stage, or hormonal therapy.

β1C Protein Expression in Neoplastic and Normal Prostate Tissues

Among the specimens showing down-regulation of β1C mRNA, 13 were selected for immunoblotting analysis of β1C and β1 integrins. Figure 7, A and B, shows the results of our immunoblotting analysis. β1C and β1 were both expressed in normal (Figure 7A, lanes 1 and 2) and in tumor prostate tissue (Figure 7A, lanes 3 to 15) as described previously.[16,20] The results in this set of specimens show a dramatic down-regulation of β1C in lysates from neoplastic tissues compared with normal tissues: β1C protein levels ranged from 8 to 28% (with an average value of 19 ± 4%) of the levels found in normal tissues (Figure 7B). In contrast, β1 protein levels were comparable in normal and prostatic adenocarcinoma tissues (Figure 7, A and B).

Discussion

We show in this study, for the first time, that β1C integrin mRNA expression is reduced in neoplastic prostate *versus* normal prostate tissues. Using the same tissue specimens, we also show down-regulation of mRNA levels of all β1 variants. At the protein level, decreased expression of β1C is observed in agreement with previous findings in prostatic adenocarcinoma as well as in other neoplasia.[20,32,33] In contrast, the protein levels of all β1 variants are comparable in normal and neoplastic prostate, thus indicating a selective down-regulation of the β1C protein.

Alterations of integrin expression in prostate cancer have been previously described by several groups.[17–22] In normal prostate, it has been shown that β1 and β4 are found in epithelial cells with redistribution to the whole surface (β1) or loss of expression (β4) associated with the malignant phenotype.[11] In this study, a selective down-regulation of β1C at the protein level is shown. β1C is one of the four known β1 variants; among these, the

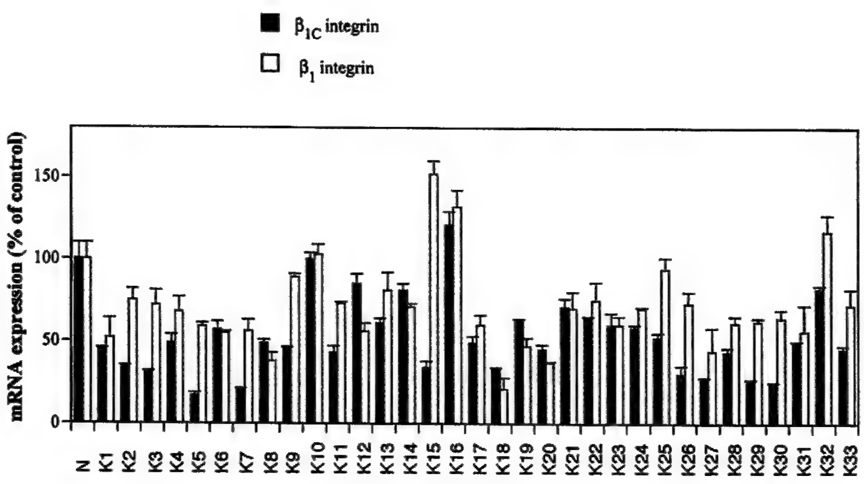

Figure 4. β1C and β1 mRNA expression in neoplastic prostate specimens. β1C and β1 mRNA expression was analyzed as described in Figures 1 and 3. The average of either β1C or β1 mRNA expression levels in five normal prostate tissue specimens (N) was set at 100. β1C and β1 mRNA levels in neoplastic prostate tissues (K1–K33) were calculated as percentage of N. Mean values ± SEM from at least three different experiments are shown.

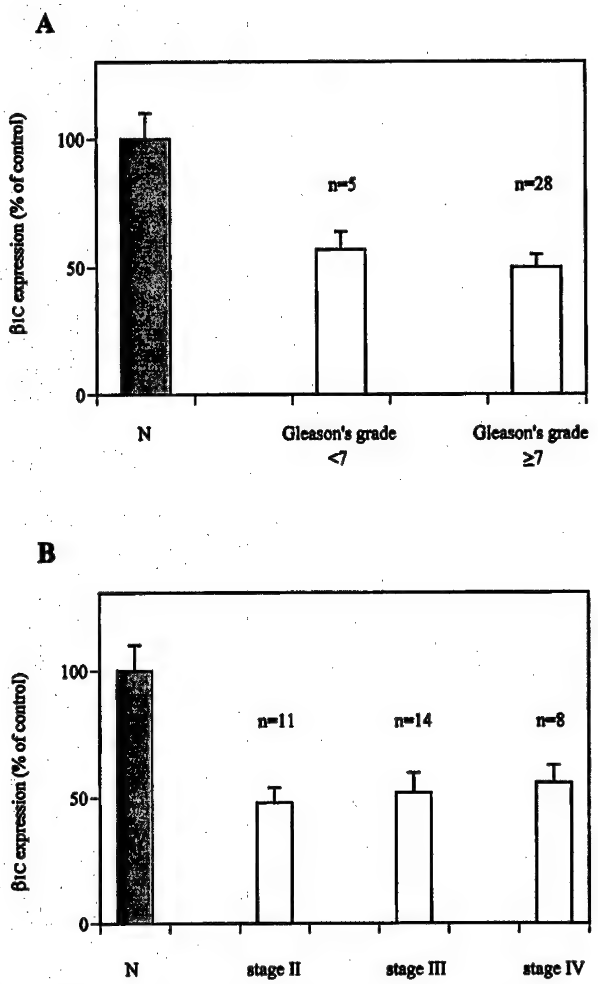

Figure 5. β1C mRNA expression does not correlate with Gleason's grade or with tumor stage in prostate carcinoma. β1C mRNA expression was evaluated as described in Figures 1 and 4: the average of β1C mRNA expression levels in five normal prostate tissue specimens (N) was set at 100. β1C mRNA levels in neoplastic prostate tissues were calculated as percentage of N. Mean values ± SEM from at least three different experiments are shown. **A:** Correlation of β1C mRNA expression with Gleason's grade. The 33 patients, described in Table 1, were divided into two groups: the first one included five patients affected by prostate carcinoma with Gleason's grade <7; the second one included 28 patients affected by prostate carcinoma with Gleason's grade ≥7. **B:** Correlation of β1C mRNA expression with tumor stage. The 33 patients were divided into three groups: the first group included 11 patients affected by stage II prostate carcinoma; the second one included 14 patients affected by stage III prostate carcinoma, and the third one included eight patients affected by stage IV prostate carcinoma. N, normal prostate tissue specimens.

β1B and β1D variants are unlikely to be found in prostate cancer tissue, because β1B is restricted to skin and liver tissues, whereas β1D is found only in striated muscle cells.[11] The two remaining β1 integrin subunits, β1C and β1A, are pathophysiologically important for cancer growth because they differentially affect cell proliferation; β1C inhibits proliferation, whereas β1A promotes it. Thus, it is conceivable that a strong pressure in prostate cancer to maintain selectively reduced mRNA and protein levels of β1C would occur. In contrast, although reduced at the mRNA level, β1 protein levels, that are likely to reflect β1A levels, are maintained constant in normal and neoplastic prostate tissues by compensatory mechanisms that remain to be identified.

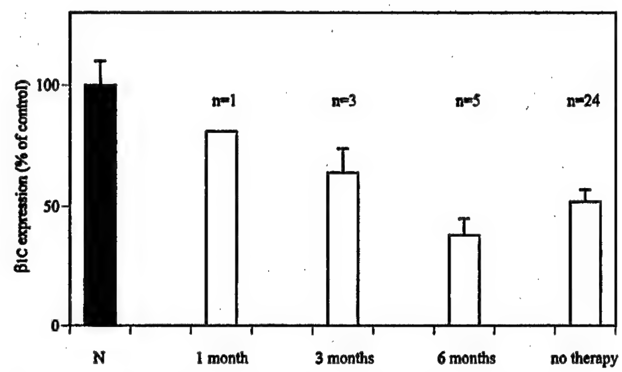

Figure 6. Hormonal therapy does not affect β1C mRNA expression. β1C mRNA expression was evaluated as described in Figures 1 and 4: the average of β1C mRNA expression levels in five normal prostate tissue specimens (N) was set at 100. β1C mRNA levels in neoplastic prostate tissues were calculated as percentage of N. Mean values ± SEM from at least three different experiments are shown. The 33 patients, described in Table 1, were divided into four groups: the first group included one patient who had undergone a 1-month therapy (1 month); the second group included three patients who had undergone 3-month therapy (3 months); the third group included five patients who had undergone 6-month therapy (6 months). The fourth group included 24 patients who had not undergone any treatment (no therapy). N, normal prostate tissue specimens.

The results reflect a specific down-regulation of β1C in prostate epithelial cells because this molecule is expressed in a cell-type-specific manner and is not found in the stroma.[20] This sophisticated regulation of the β1C variant in prostate cancer suggests a tight control at the transcriptional and/or translational level of its expression to prevent inhibition of prostate epithelial cell proliferation. It is conceivable that either a transcriptional or a posttranscriptional regulation of β1C expression might be responsible for the decreased mRNA levels, whereas at

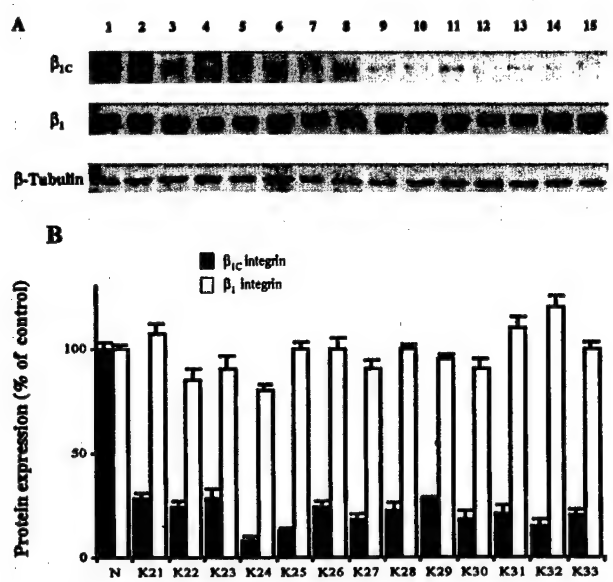

Figure 7. β1C and β1 protein expression in prostatic adenocarcinoma. **A:** Either normal (**lanes 1** and **2**) or tumor (**lanes 3–15**) tissue detergent extracts were electrophoresed on 7.5% SDS-polyacrylamide gel electrophoresis under reducing conditions, and immunostained using antibody either to β1C integrin or to β1 integrin or to β-tubulin as indicated in Materials and Methods. **B:** The mean values ± SEM of either β1C or β1 levels in neoplastic tissues, normalized using β-tubulin, were calculated as percentage of the levels detected in two normal prostate tissues (N). At least four separate measurements for each specimen were performed.

the protein level, translation activities or protein degradation that are expected to be increased in cancer cells, might be accounted responsible for the decreased β1C protein levels. *In vitro*, regulation of integrin mRNA and protein expression has been shown to be modulated by the protein kinase C activator PMA. PMA was shown to stimulate adhesion of tumor cells to fibronectin and fibrinogen by modulating αIIbβ3 expression in human prostatic adenocarcinoma cells.[21] Moreover, PMA determined changes of αV mRNA expression in leukemia cells[34] and caused increase of α2 integrin cell surface expression in tumor progression by enhancing α2 integrin transcription.[35] It is conceivable that protein kinase C might act also as a modulator of β1C integrin expression *in vitro* and *in vivo*, and this remains to be investigated. Androgen-deprivation therapy did not seem to interfere with β1C integrin expression, thus indicating that androgen-mediated mechanisms act through pathways that do not involve β1C. Since, in addition to androgens, mitogens regulate cell proliferation and integrin expression, they may be important autocrine-paracrine modulators of the neoplastic phenotype and of β1C expression *in vivo*.[14,36–40]

Because β1C is a spliced variant of the β1 integrin subfamily, down-regulation of its mRNA could have been a reflection of an altered splicing mechanism occurring in prostate cancer. Our data show that altered splicing mechanisms are unlikely to explain the reduced β1C mRNA levels in prostate cancer because all β1 integrin mRNAs were found down-regulated. In this regard, Tamura et al[41] and Belkin et al[42] have demonstrated that splicing mechanisms control specific integrin expression at different stages of differentiation; our results show that β1C mRNA and protein levels are down-regulated in differentiated (low to intermediate Gleason's grade) as well as poorly differentiated (high Gleason's grade) prostate cancer and are not regulated in a differentiation-dependent manner.

Consistent with our recent experimental evidence ruling out a role for β1C cytoplasmic domain in $\alpha\beta$ heterodimer assembly or in determining ligand specificity but affecting selectively intracellular signaling,[43] our findings suggest that the regulated expression of different integrin-variant cytoplasmic domains might provide a highly specialized mechanism to control cell proliferation and intracellular signaling pathways in normal and pathological conditions. Recent studies have demonstrated that the β1C variant, using a unique signaling mechanism, selectively inhibits the mitogen-activated protein kinase pathway by preventing Ras activation without affecting either survival signals stimulated by integrins or cellular interactions with the extracellular matrix.[43] Specifically, in human prostate epithelial cells, β1C is coexpressed with the cell cycle inhibitor p27^{kip1}, the loss of which correlates with poor prognosis in prostate cancer;[16] furthermore, exogenous expression of β1C *in vitro* inhibits prostate cell proliferation, and is accompanied by an increase in p27^{kip1}.[16] These findings suggest a role for β1 specific cytodomain sequences in maintaining an intracellular balance of proliferation and survival signals and point to β1C as an upstream regulator of p27^{kip1} expression and, therefore, as a potential target for tumor suppression in prostate cancer.

Efforts to confirm the prognostic value of β1C are in progress. Future studies will indicate whether loss of β1C expression in association with other traditional or novel markers has greater prognostic potential than each factor alone.

Acknowledgment

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$p27^{kip1}$ acts as a downstream effector of and is coexpressed with the β_{1C} integrin in prostatic adenocarcinoma

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Integrins are a large family of transmembrane receptors that, in addition to mediating cell adhesion, modulate cell proliferation. The β_{1C} integrin is an alternatively spliced variant of the β_1 subfamily that contains a unique 48-amino acid sequence in its cytoplasmic domain. We have shown previously that *in vitro* β_{1C} inhibits cell proliferation and that *in vivo* β_{1C} is expressed in nonproliferative, differentiated epithelium and is selectively downregulated in prostatic adenocarcinoma. Here we show, by immunohistochemistry and immunoblotting analysis, that β_{1C} is coexpressed in human prostate epithelial cells with the cell-cycle inhibitor $p27^{kip1}$, the loss of which correlates with poor prognosis in prostate cancer. In the 37 specimens analyzed, β_{1C} and $p27^{kip1}$ are concurrently expressed in 93% of benign and 84%–91% of tumor prostate cells. Forced expression of β_{1C} *in vitro* is accompanied by an increase in $p27^{kip1}$ levels, by inhibition of cyclin A–dependent kinase activity, and by increased association of $p27^{kip1}$ with cyclin A. β_{1C} inhibitory effect on cell proliferation is completely prevented by $p27^{kip1}$ antisense, but not mismatch oligonucleotides. β_{1C} expression does not affect either cyclin A or E levels, or cyclin E–associated kinase activity, nor the mitogen-activated protein (MAP) kinase pathway. These findings show a unique mechanism of cell growth inhibition by integrins and point to β_{1C} as an upstream regulator of $p27^{kip1}$ expression and, therefore, a potential target for tumor suppression in prostate cancer.

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Introduction

Integrins are a large family of transmembrane receptors composed of an α and a β subunit that, in addition to mediating cell adhesion to the extracellular matrix (ECM), have been shown to regulate cell growth, survival, and differentiation (1, 2). Considerable effort has been devoted to elucidate the intracellular signaling events modulated by integrins, in particular the activation of intracellular protein kinases, including members of the mitogen-activated protein (MAP) kinase family (3–5). The MAP kinase family is composed of serine/threonine kinases that, in addition to integrin engagement, are activated by mitogens and modulate gene expression (4) and, ultimately, cell proliferation.

The β_{1C} integrin is an alternatively spliced variant of the β_1 subfamily that contains a unique 48-amino acid sequence in its cytoplasmic domain (6). *In vivo*, β_{1C} is expressed in nonproliferative and differentiated epithelium (7). In the prostate, β_{1C} is found in benign glandular epithelial cells and is selectively downregulated in adenocarcinoma (8). Previous studies have shown that forced expression of either β_{1C} or its cytoplasmic domain inhibits proliferation of both tumorigenic (specifically PC3 prostate cancer and Chinese hamster ovary [CHO]) and nontumorigenic (specifically 10T1/2) cells (7, 9, 10) without affecting cytoskeletal or focal adhesion organization (10).

It is well established that cell-cycle progression is regulated by cyclin-dependent kinases (CDKs) (11), whose activity is controlled by cyclin binding, phosphorylation/dephosphorylation, and association with a group of CDK-inhibitory proteins, designated CKIs (12). A member of a CKI family, $p27^{kip1}$, controls cell-cycle progression by specific binding to cyclin D-, E-, and A-CDK complexes. This inhibitor is highly expressed in nonproliferative, quiescent cells and its levels are increased by growth-inhibitory signals (12). Furthermore, its forced overexpression is sufficient to inhibit cell proliferation (12). The pathophysiological relevance of $p27^{kip1}$ regulated expression is suggested by recent studies showing that in prostate cancer, as well as in breast or colorectal carcinomas, loss of $p27^{kip1}$ is an adverse prognostic factor that correlates with poor patient survival (13–17).

Several studies have shown that cell adhesion to the ECM is required for cell-cycle progression and proliferation in different cell types (18). Loss of cell anchorage to the ECM recently has been shown to upregulate the expression of $p27^{kip1}$ and $p21^{cip1/waf1}$, while at the same time decreasing the levels of cyclin A (19–21). Some studies have also indicated that the expression of cyclin D1 and E is adhesion dependent (19, 21, 22). Anchorage is also required for cyclin E-CDK2 and cyclin D-CDK4/6 activi-

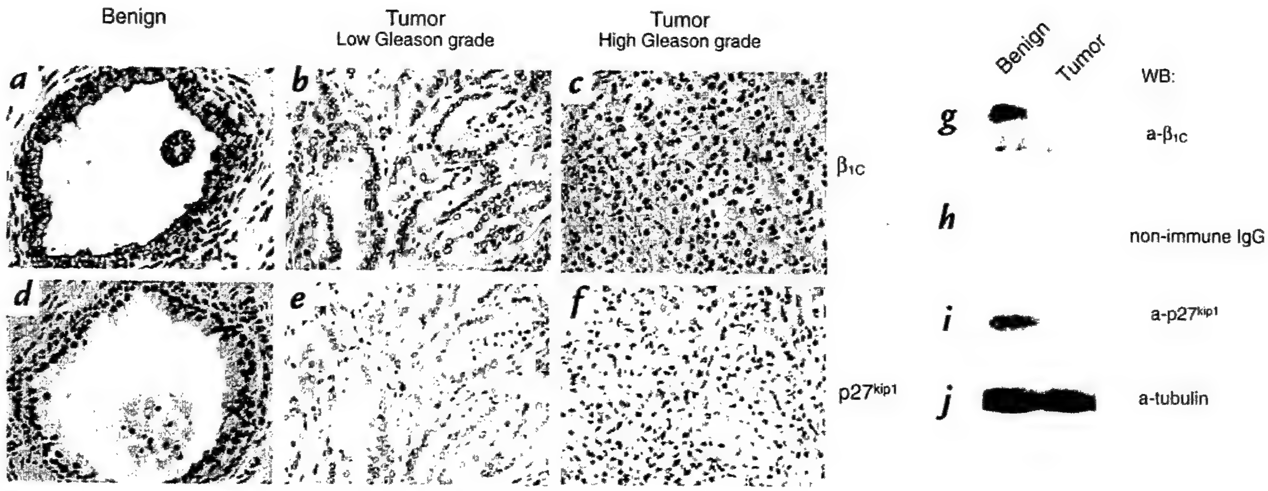

Figure 1

(*a–j*) Downregulation of β_{1C} and p27^{kip1} expression in prostatic adenocarcinoma. The expression of β_{1C} and p27^{kip1} in a representative case of benign (*a* and *d*) or neoplastic (*b*, *c*, *e*, and *f*) prostate tissue was evaluated by immunohistochemistry (*a–f*) using 1.8 μg/ml affinity-purified antibody to β_{1C} (*a–c*) or 0.6 μg/ml monoclonal antibody to p27^{kip1} (*d–f*), and by immunoblotting (*g–j*). Tumor or benign prostate tissue detergent extracts were electrophoresed on 10% SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes, and immunostained using 2 μg/ml affinity-purified antibody to β_{1C} (*g*), 2 μg/ml nonimmune rabbit IgG as negative control for β_{1C} (*h*), or 0.8 μg/ml monoclonal antibody to p27^{kip1} (*i*). Monoclonal antibody to tubulin was used to control for protein loading (*j*). Proteins were viewed by ECL. *ECL*, enhanced chemiluminescence. The results show that β_{1C} and p27^{kip1} expressions correlate in benign and neoplastic prostate.

ties (20, 21, 23). Changes of p27^{kip1}, p21$^{cip1/waf1}$, and cyclin A expression levels, as well as cyclin E-CDK2 activity, were also observed in response to structural alterations of collagen matrices and consequent intracellular modifications of cytoskeleton and focal adhesion sites (24). Overall, these studies show that control of cell-cycle molecule expressions and activities is mediated by adhesion- and spreading-dependent events. At this time, however, modulation by integrins of either p27^{kip1} expression or CDK activities in the absence of changes in cell adhesion or spreading has never been shown.

In this study, we show that *in vivo* β_{1C} and p27^{kip1} expressions are concurrently downregulated in neoplastic prostate epithelial cells, thus describing for the first time an *in vivo* correlation of expression of integrins and a cell-cycle inhibitor. We hypothesized that β_{1C} may function as an upstream regulator of specific CKIs and would increase p27^{kip1} levels to inhibit cell proliferation. We show that *in vitro* forced expression of β_{1C} is accompanied by an increase in p27^{kip1} levels and in its association with cyclin A, and by selective inhibition of cyclin A–dependent, but not cyclin E–dependent, kinase activity. Moreover, p27^{kip1} antisense, but not mismatch, oligonucleotides prevented inhibition of cell proliferation observed in β_{1C} transfected cells. The study also shows that neither cyclin A nor E expressions, nor the Ras/MAP kinase pathway are affected. These data describe a unique mechanism of cell growth inhibition by integrins and point to β_{1C} as an upstream regulator of p27^{kip1} expression and, therefore, a target molecule for tumor suppression in prostate cancer.

Methods

Reagents. Rabbit affinity-purified antibodies specific for the β_{1C} subunit cytoplasmic domain were generated and affinity-purified as described previously (8). The following antibodies were used: mouse monoclonal antibodies (MABs) to p27^{kip1} and to p130Cas (Transduction Laboratories, Lexington, Kentucky, USA); to β-tubulin (Sigma Chemical Co., St. Louis, Missouri, USA); Ha2/5 to rat β_1 integrin (PharMingen, San Diego, California, USA); TS2/16 to human β_1 integrin extracellular domain purchased from American Type Culture Collection (Rockville, Maryland, USA) and a kind gift of M.E. Hemler (Dana-Farber Cancer Institute, Boston, Massachusetts, USA); and 12CA5 to hemagglutinin (American Type Culture Collection). Also used were: rabbit affinity-purified antibodies to cyclin E, to cyclin A, and to extracellular signal-regulated kinase-1 and -2 (ERK-1 and -2; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA); rabbit antisera to cyclin A and to CDK2, kindly provided by H. Zhang (Yale University, New Haven, Connecticut, USA); and nonimmune rabbit IgG (Sigma Chemical Co.). Human plasma fibronectin was purified by affinity chromatography on gelatin-Sepharose (25). Human vitronectin was purchased from Life Technologies Inc. (Gaithersburg, Maryland, USA).

Tissue specimens and immunohistochemistry. Specimens from 37 radical prostatectomies, performed for prostatic adenocarcinoma at the Yale-New Haven Hospital (New Haven, Connecticut, USA), were included in this study according to a protocol approved by Yale University School of Medicine Review Board. Hematoxylin and eosin–stained sections were reviewed, and sections showing both neoplastic and benign prostate tissue were selected for evaluation of β_{1C} and p27^{kip1} immunoreactivity. Serial sections from paraffin-embedded and formalin-fixed tissue specimens stained previously using antibodies to β_{1C} were selected (8). Single-labeling experiments were performed as described previously (7, 8). For double-labeling experiments, tissue sections were first stained using MAB to p27^{kip1} and then treated sequentially with a biotinylated horse anti-mouse secondary antibody (Vector Laboratories, Burlingame, California, USA) and peroxidase-labeled streptavidin (Boehringer Mannheim, Indianapolis, USA). Development of peroxidase activity was achieved using 3,3′-diaminobenzidine tetrahydrochloride dehydrate (Sigma Chemical Co.) as chromogen. After p27^{kip1} immunodetection, tissue sections were rinsed in distilled water and stained sequentially with rabbit affinity-purified antibody to β_{1C}, a

Table 1

Correlation of β_{1C} and p27^{kip1} expression in benign and neoplastic prostate

Benign				Tumor			
n*	%	β_{1C}	p27^{kip1}	n*	%	β_{1C}	p27^{kip1}
37	93 ± 5	+	+	7	84 ± 5.3	+	+
				24	91 ± 9.4	–	–
				1	0	+	–

Adjacent areas of neoplastic and benign prostate tissue were stained in 32 of the 37 analyzed specimens, whereas only benign glands were found in the remaining five specimens. Immunoreactivity of either β_{1C} or p27^{kip1} in benign and malignant cells was evaluated as positive (+) if more than 30% of the cells were stained, and as negative (–) if less than 30% of the cells were stained. The correlation of β_{1C} and p27^{kip1} expression is highly significant, as evaluated by Fisher exact test ($P < 0.0001$). *Number of analyzed cases.
% Mean percentage of cells showing concurrent expression of β_{1C} and p27^{kip1}, as evaluated in double immunostaining experiments. The percentage of cells that showed correlation of β_{1C} and p27^{kip1} expression was calculated as ratio of number of cells either expressing or lacking both molecules/total cell number counted in five fields ×100.

biotinylated goat anti-rabbit secondary antibody (Vector Laboratories), and the alkaline phosphatase–labeled streptavidin (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Maryland, USA), followed by alkaline phosphatase substrate kit III (Vector Laboratories). The slides were then rinsed in distilled water and mounted using Aqua Mount (Lerner Laboratories, Pittsburgh, Pennsylvania, USA) without dehydration.

Immunostaining evaluation. Adjacent areas of neoplastic and benign prostate tissue from the same section were evaluated essentially as described (13, 14). The β_{1C} and p27^{kip1} immunoreactivity, in double-staining experiments, was assessed independently by three investigators (M. Fornaro, G. Tallini, and L.R. Languino) and scoring was performed in a blinded manner. Five high-power fields were randomly chosen and scored for the percentage of cells either showing or lacking β_{1C} and p27^{kip1} staining; a minimum of 300 cells per specimen were evaluated. β_{1C} and p27^{kip1} expression in benign or neoplastic cells was scored as positive (+) if more than 30% of the cells were stained and as negative (–) if less than 30% of the cells were stained. The percentage of cells that showed correlation of β_{1C} and p27^{kip1} expression was calculated as the ratio of number of cells either expressing or lacking both molecules/total cell number counted in five fields (×100). There was 98% concordance among the observers' scores; in one instance, because of disagreement among the observers, the specimen was discarded. Statistical analysis was performed using Fisher's exact test. In double-staining experiments, hematoxylin counterstain was not used.

Cells and transfections. Normal nonimmortalized rat prostate epithelial cells, NRP152 (26), were maintained in DMEM-F12 (Life Technologies Inc.) supplemented with 5% FCS (Gemini Bioproducts Inc., Calabasas, California, USA), 2 mM glutamine (Gemini Bioproducts Inc.), 20 ng/ml epidermal growth factor (EGF) (R&D Systems Inc., Minneapolis, Minnesota, USA), 5 μg/ml insulin (Sigma Chemical Co.), 0.1 μM dexamethasone (Sigma Chemical Co.), and 10 ng/ml cholera toxin (Sigma Chemical Co.). CHO cells (American Type Culture Collection) were maintained in DMEM (Life Technologies Inc.) supplemented with 10% FCS, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin (Gemini Bioproducts Inc.), and 0.1 mM nonessential amino acids (Life Technologies Inc.).

The tetracycline-regulated expression system, designed for the inducible expression of exogenous proteins in mammalian cells, consists of two plasmids: the pTet-tTA plasmid contains the neomycin-resistance gene and encodes a transcriptional transactivator (tTA) that drives expression of itself and a target gene inserted into the multiple cloning site of the second plasmid, pTet-Splice (27). To obtain stable transfectants expressing β_{1C} in a tetracycline-regulated system, *ClaI-XbaI* fragment–encoding full-length human β_{1C} was isolated from Bluescript-β_{1C} (9) and subcloned into *ClaI-SpeI* sites in the pTet-Splice plasmid, a kind gift of D. Schatz (Yale University), to generate the pTet-β_{1C} construct. NRP152 cells were electroporated using a Genepulser apparatus (Bio-Rad Laboratories Inc., Hercules, California, USA) set at 300 V and 950 μF, using either 20 μg pTet-β_{1C} or pTet-splice, along with 10 μg pTet-tTA. Neomycin-resistant cells were selected using medium containing 0.56 mg/ml G418 (Life Technologies Inc.). G418-resistant clones were isolated in two rounds and screened for cell surface expression of β_{1C} integrin by FACS®, using TS2/16, MAB against human β_1 integrin, or 12CA5 MAB, as a negative control, as described (9). Stable transfectants were maintained in growth medium containing 1 μg/ml tetracycline (Boehringer Mannheim) and 0.1 mg/ml G418.

The CHO cells were transfected as described above, either with the pTet-β_{1C} construct or the pTet-splice, along with the pTet-tTA plasmid by electroporation, using a Genepulser apparatus set at 350 V and 950 μF. Neomycin-resistant cells were selected using medium containing 1.4 mg/ml G418. G418-resistant clones were isolated in two rounds and screened for cell surface expression of the human β_{1C} integrin by FACS® analysis, and stable transfectants were maintained as described above.

The CHO cells were also transiently transfected by electroporation using pBJ1-β_{1C}, or pBJ1-β_{1A}, or pBJ1 vector alone, and surface expression of the transfected β_{1C} or β_{1A} integrins was evaluated by FACS® analysis as described (9).

CHO cell adhesion to fibronectin (3 μg/ml), vitronectin (30 μg/ml), BSA (10 mg/ml; Sigma Chemical Co.), and TS2/16 (1:10 dilution of culture supernatant) was performed as described previously (28) using [^{51}Cr]-labeled (Du Pont Nen Research Products, Wilmington, Delaware, USA) cells.

Immunoblotting, immunoprecipitation, and in vitro kinase assay. NRP152 transfectants were cultured for 72 h in the absence of tetracycline, then cells were detached with 0.05% trypsin/0.53 mM EDTA (Life Technologies Inc.), washed three times, and resuspended in serum-

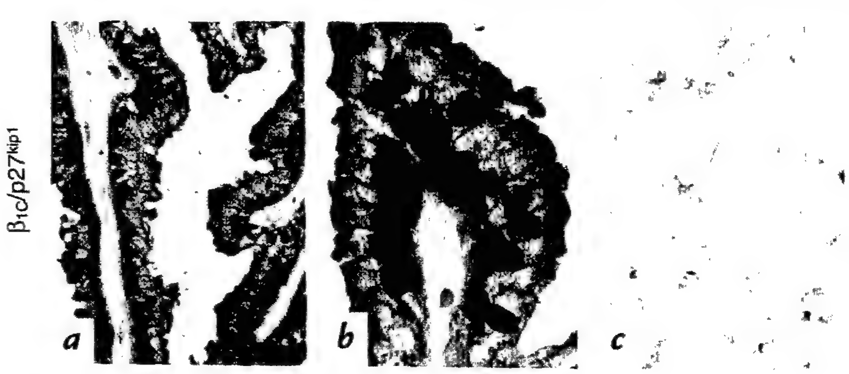

Figure 2

(*a–c*) Coexpression of β_{1C} and p27^{kip1} in benign and neoplastic prostate tissue. Double staining for β_{1C} and p27^{kip1} is shown in a representative case of benign (*a* and *b*) or intermediate Gleason's score neoplastic (*c*) prostate tissue (blue and dark brown staining for β_{1C} and p27^{kip1}, respectively). Immunohistochemical analysis was performed as in Fig. 1. Hematoxylin counterstain was not used. The results show that β_{1C} and p27^{kip1} expressions correlate in benign and neoplastic prostate.

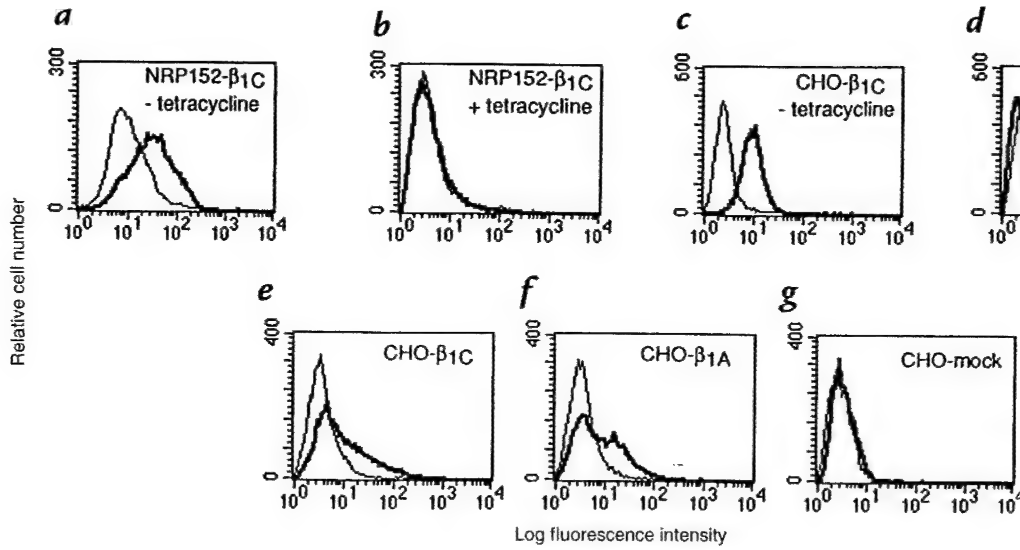

Figure 3

(*a*–*g*) Exogenous expression of β_{1C} in NRP152 and CHO cell transfectants. Stable cell transfectants expressing β_{1C} were generated using a tetracycline-regulated expression system. NRP152-β_{1C} or CHO-β_{1C} stable cell transfectants were cultured for 72 h, either in the absence (*a* and *c*) or in the presence (*b* and *d*) of 1 μg/ml tetracycline and analyzed by FACS® using TS2/16, MAB to human β_1 integrin, or 12CA5 as a negative control, followed by FITC-goat anti-mouse IgG. Fluorescence intensity is expressed in arbitrary units. FACS® analysis of a representative β_{1C} clone is shown. CHO cells were transiently transfected using pBJ1-β_{1C} (*e*), or pBJ1-β_{1A} (*f*), or pBJ-1 vector (*g*), and after 44 h, cells were stained and analyzed as described above. *Thick line*, TS2/16; *thin line*, 12CA5. *MAB*, monoclonal antibody.

free medium. To engage β_1 integrins, NRP152-β_{1C} transfectants were seeded on tissue culture dishes coated with TS2/16, whereas NRP152-mock transfectants were seeded on tissue culture dishes coated with Ha2/5 for 1 h at 37°C, washed three times with serum-free medium, and cultured for 20 h in growth medium. Cells were then lysed, and $p27^{kip1}$ expression was evaluated by immunoblotting as described below.

To detect cyclin E, cyclin A, CDK2 or $p27^{kip1}$ stable NRP152 or CHO cell transfectants were lysed with Nonidet P-40 (NP-40) lysis buffer: with 0.5% NP-40 (Calbiochem, San Diego, California, USA), 150 mM NaCl, 50 mM HEPES (pH 7.5), 10% glycerol, 0.1 mM sodium vanadate (Sigma Chemical Co.), 1 mM sodium fluoride (Sigma Chemical Co.), 1 mM PMSF (Life Technologies Inc.), 10 μg/ml aprotinin (Sigma Chemical Co.), 10 μg/ml leupeptin (Calbiochem) for 30 min at 4°C (29). Transiently transfected CHO cells were lysed in 0.1% Tween-20 (American Bioanalytical, Natick, Massachusetts, USA), 150 mM NaCl, 50 mM HEPES (pH 7.5), 10% glycerol, 1 mM EDTA, 2.5 mM EGTA, 0.1 mM sodium vanadate, 1 mM sodium fluoride, 1 mM DTT (Bio-Rad Laboratories Inc.), 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and sonicated (30). Similar results were observed using either NP-40 or Tween-20 lysis buffers (29, 30). To analyze β_{1C} and $p27^{kip1}$ expression in the prostate, either benign or tumor frozen tissue specimens obtained from radical prostatectomies were homogenized in lysis buffer containing 100 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100 (Sigma Chemical Co.), 5% SDS, 1 mM PMSF, 10 μg/ml leupeptin, 1 mM benzamidine (Sigma Chemical Co.), 1 μM D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) (Boehringer Mannheim), 10 μg/ml soybean trypsin inhibitor (Life Technologies Inc.), using an OMNI 2000 homogenizer (OMNI International Inc., Gainesville, Virginia). The protein content in each lysate was quantitated using the bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Co., Rockford, Illinois, USA). Immunoblotting of cyclin E, cyclin A, CDK2, $p27^{kip1}$, and tubulin was performed as described (31); immunoblotting of β_{1C} integrin was carried out as described (8).

Cyclin A or cyclin E were immunoprecipitated (31), and the associated kinase activities were assayed as described (30), using 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Life Sciences Inc., Arlington Heights, Illinois, USA) and 33 μg/ml histone H1 (Life Technologies Inc.) as a substrate. Phosphorylated histone H1 was observed using autoradiography.

The association of cyclin A with $p27^{kip1}$ was detected by immunoprecipitation with rabbit antiserum to cyclin A, followed by immunoblotting with MAB to $p27^{kip1}$, as described (31). In each immunoprecipitation, the applied amounts of cyclin A from each lysate were comparable, as evaluated by densitometric analysis.

In all instances, quantification of immunoreactive bands was performed by densitometric analysis; the values were normalized for protein loading and reported as mean ± SEM. Group differences were compared using Student's *t* test.

Oligonucleotide treatment and proliferation assay. CHO-β_{1C} cell transfectants (3.5×10^3) were plated on tissue culture plates in growth medium containing 1 μg/ml tetracycline. After approximately 24 h, tetracycline was removed to induce β_{1C} expression. A mixture of 30 nM oligonucleotides (Gilead Sciences, Foster City, California, USA) (32) and 5 μg/ml GS3815 cytofectin (Gilead Sciences) in OPTI-MEM I (Life Technologies Inc.) was incubated for 15 min at room temperature and added to CHO cell transfectants for 24 h. Cells were rinsed three times with PBS and cultured for 48 h at 37°C, either in the absence or in the presence of 10% FCS, and pulsed with 1 μCi [^{3}H]thymidine per well (5.0 Ci/mmol; Amersham Life Sciences Inc.) during the last 3 h of the 48-h culture. [^{3}H]thymidine incorporation was evaluated as described (9). In each experiment, duplicate or triplicate observations were performed, and the values are reported as mean ± SEM. Group differences were compared using one-way analysis of variance.

By using FITC-labeled oligonucleotides as described (32), we determined that 90%–95% of the cells were positive for oligonucleotide uptake. The sequences of the antisense and mismatch $p27^{kip1}$ C-5-propyne-modified phosphorothioates used were 5′-UGGCUCUCCUGCGCC-3′ and 5′-UCCCUUUGGCGCGCC-3′, respectively (32).

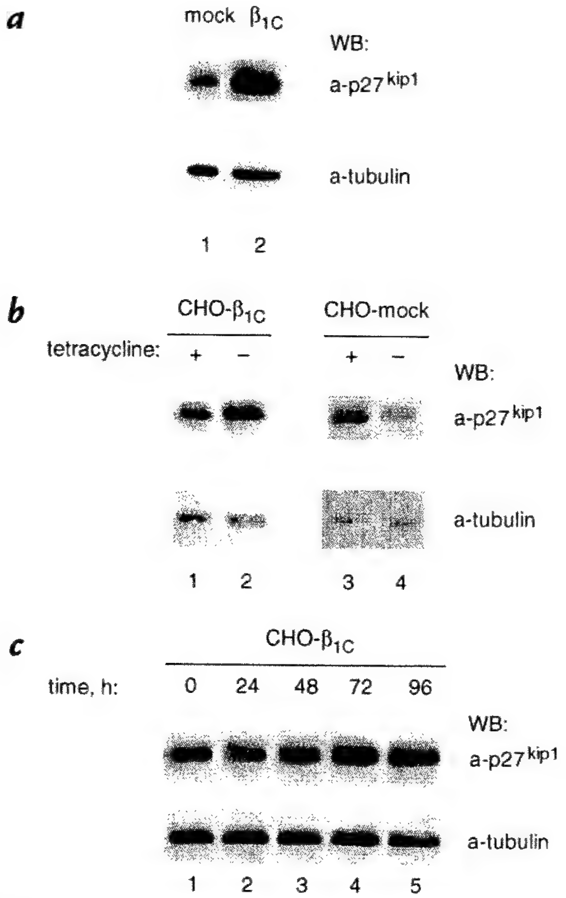

Figure 4
β_{1C} expression is accompanied by increased $p27^{kip1}$ protein levels in NRP152 and CHO cells. (***a***) NRP152-β_{1C} (lane *2*) or NRP152-β_{1C}-mock (lane *1*) stable cell transfectants were cultured for 72 h in the absence of tetracycline, detached, and NRP152-β_{1C} was seeded on tissue culture dishes coated with TS2/16, whereas NRP152-mock transfectants were seeded on tissue culture dishes coated with Ha2/5 for 1 h at 37°C, washed three times with serum-free medium, and cultured for 20 h in growth medium. Cells were then lysed and $p27^{kip1}$ expression levels were evaluated by immunoblotting using 0.8 μg/ml MAB to $p27^{kip1}$ (*top*). (***b*** and ***c***) CHO-β_{1C} (*b*, lanes *1* and *2*; *c*) or CHO-β_{1C}-mock (*b*, lanes *3* and *4*) stable cell transfectants were cultured for 72 h in *b* and for the indicated times in *c*, either in the absence (*b*, lanes *2* and *4*; *c*) or in the presence (*b*, lanes *1* and *3*) of 1 μg/ml tetracycline. In these experiments cells were not detached and allowed to reattach to MAB to β_1 integrins, but were lysed in the tissue culture plate, and $p27^{kip1}$ expression levels were evaluated as described in *a*. The experiments were repeated three times using two different β_{1C} clones with similar results. Group differences were compared using Student's *t* test. The differences in $p27^{kip1}$ expression levels in CHO-β_{1C}, but not in CHO-mock transfectants in the presence or in the absence of tetracycline, are statistically significant ($P = 0.03$). Control for protein loading was provided by MAB to tubulin (*a–c*, *bottom*). Proteins were viewed by ECL. Time refers to the length of time in absence of tetracycline.

MAP kinase mobility shift and immunocomplex kinase assay. Serum-starved CHO cells, transiently transfected either with β_{1C} or β_{1A} integrins, were detached with 0.05% trypsin/0.53 mM EDTA. After trypsin neutralization by 1 mg/ml soybean trypsin inhibitor, cells were washed twice and resuspended in serum-free medium containing 2% BSA. Cells were incubated for 30 min at 37°C, then kept in suspension or plated on tissue culture plates coated with TS2/16, antibody to β_1 integrin, either in the absence or in the presence of 1% FCS, or with 10 μg/ml fibronectin in the absence of FCS for 10 min at 37°C. Cell lysis, immunoblotting, and kinase assay were carried out as described (33).

Results

Concurrent expression of β_{1C} and $p27^{kip1}$ in prostatic adenocarcinoma. Expression of β_{1C} and $p27^{kip1}$ was examined by immunohistochemistry and immunoblotting in 37 specimens resected for prostatic adenocarcinoma (Figs. 1 and 2; Table 1). Marked expression of both β_{1C} and, in the nuclei, of $p27^{kip1}$ was consistently observed in benign glandular epithelial cells (Fig. 1, *a* and *d*); whereas downregulation of both molecules was observed in neoplastic tissue (Fig. 1, *b*, *c*, *e*, and *f*). Double-staining experiments showed a very high correlation of β_{1C} and $p27^{kip1}$ expression in 93% of benign cells (Fig. 2, *a* and *b*; Table 1) and in 84%–91% of neoplastic cells of the 37 specimens analyzed (Fig. 2*c* and Table 1). Both β_{1C} and $p27^{kip1}$ were downregulated in tumor areas compared with benign counterparts in 24 cases (75% of the specimens analyzed). They were coexpressed in benign and tumor areas in 7 cases (22% of the specimens analyzed). In only one instance did their expression not correlate (3% of the specimens analyzed; Table 1). Among the 24 specimens showing downregulation of both β_{1C} and $p27^{kip1}$, two were selected for immunoblotting analysis. The results confirmed downregulation of both molecules in the lysates from both neoplastic tissues compared with their benign counterparts (Fig. 1, *g* and *i*; data not shown).

Forced β_{1C} expression is accompanied by increased levels of $p27^{kip1}$. On the basis of these observations, we hypothesized that β_{1C} might have a causal role in regulating $p27^{kip1}$ levels *in vitro*. To test this hypothesis, we generated NRP152 or CHO transfectants expressing human β_{1C} under the control of a tetracycline-regulated promoter. β_{1C} expression in NRP152 (Fig. 3, *a* and *b*) or CHO (Fig. 3, *c* and *d*) cells was analyzed by fluorescence-activated cell sorter (FACS®) using either TS2/16, MAB to human β_1 integrin (which does not cross-react with either rat or hamster β_1 integrin), or 12CA5 as a negative control; in both cell types, maximal β_{1C} expression was obtained 72 hours after tetracycline removal (not shown). NRP152-β_{1C} stable transfectants were detached and seeded on tissue culture plates coated with TS2/16, whereas mock-transfected cells were plated on Ha2/5, MAB to rat β_1 integrin. Immunoblotting analysis showed an increase of $p27^{kip1}$ expression in NRP152-β_{1C} stable transfectants compared with mock-transfected cells (10.9 ± 2.6-fold increase in two experiments; Fig. 4 *a*, *top*). Similar results were confirmed using CHO-β_{1C} stable transfectants. In these experiments, cells were not detached and were allowed to reattach to monoclonal antibodies to β_1 integrins, but they were lysed in the tissue culture plate. In these cells, we observed a 4.65 ± 0.65-fold increase in three experiments 72 hours after removal of tetracycline (Fig. 4 *b*, compare lanes *1* and *2*; *top*) whereas mock-transfected cells showed no increase in the absence of tetracycline (1.28 ± 0.41-fold increase in three experiments; Fig. 4 *b*, compare lanes *3* and *4*; *top*). The increase of $p27^{kip1}$ levels in response to β_{1C} expression was time dependent and was observed as early as 48 hours after withdrawal of tetracycline (Fig. 4 *c*, lane *3*), with a maximum at 72

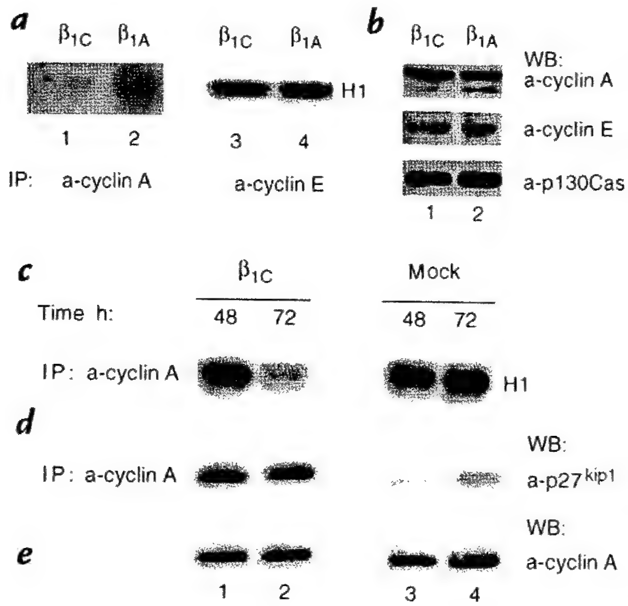

Figure 5
Cyclin A–associated kinase activity is inhibited in CHO-β_{1C} cell transfectants. (*a*) CHO cells were transiently transfected using pBJ1-β_{1C} (lanes *1* and *3*) or pBJ1-β_{1A} (lanes *2* and *4*). Total cell lysates, obtained as described in Methods, were immunoprecipitated using 1 μg/ml rabbit affinity-purified antibodies to cyclin A (lanes *1* and *2*) or to cyclin E (lanes *3* and *4*), and the associated kinase activity was assayed *in vitro* using histone H1 as a substrate. Phosphorylated histone H1 was observed by autoradiography. The experiments were repeated two to five times with consistent results. (*b*) β_{1C} expression does not affect cyclin E or cyclin A protein levels. Total cell lysates, obtained as described in *a*, were immunoblotted with 1 μg/ml rabbit affinity-purified antibodies to cyclin A (*top*) or to cyclin E (*middle*). Control for protein loading was provided by MAB to p130Cas (*bottom*). The experiments were repeated at least twice with consistent results. (*c–e*) CHO-β_{1C} (lanes *1* and *2*) or CHO-mock (lanes *3* and *4*) stable cell transfectants were cultured in the absence of tetracycline for the indicated times. In *c*, total cell lysates were immunoprecipitated using rabbit antiserum to cyclin A, and the associated kinase activity was assayed as described above. The experiments were repeated twice using two different β_{1C} clones with similar results. (*d*) Expression of β_{1C} is accompanied by increased $p27^{kip1}$ association with cyclin A. Total cell lysates were immunoprecipitated using rabbit antiserum to cyclin A, and the associated $p27^{kip1}$ was detected by immunoblotting. (*e*) Total cell lysates were immunoblotted with rabbit affinity-purified antibody to cyclin A, as described above. In *d* and *e*, the experiments were repeated three times using two different β_{1C} clones with similar results. In *b*, *d*, and *e*, proteins were viewed by ECL. Group differences were compared using Student's *t* test. In *a* and *c*, the differences between cyclin A-CDK activity in CHO-β_{1C} versus CHO-β_{1A} transfectants are statistically significant (in *a*, $P = 0.0001$; in *c*, $P = 0.0069$).

hours (Fig. 4 *c*, lane *4*). The results show that β_{1C} expression is accompanied by increased levels of $p27^{kip1}$ in both nontumorigenic as well as tumorigenic cells.

Selective inhibition of cyclin A–dependent kinase activity and increased $p27^{kip1}$ association with cyclin A in β_{1C} transfectants. Because $p27^{kip1}$ binds to and inhibits the activity of cyclin-CDK complexes (12), we investigated whether specific cyclin-associated kinase activities were inhibited in β_{1C} transfectants. Either transient transfectants or two independent stable clones expressing β_{1C} were used as controls for potential clonal variability. Cyclin A or cyclin E were immunoprecipitated from CHO cell lysates transiently expressing β_{1C} or β_{1A} integrins (Fig. 3, *e* and *f*), and the complexes were tested for their ability to phosphorylate histone H1. Cyclin A–associated kinase activity was reduced (53 ± 3% decrease in five experiments) in β_{1C} transfectants as compared with β_{1A} transfectants (Fig. 5*a*, compare lanes *1* and *2*), whereas cyclin E–associated kinase activity was unaffected (1.5 ± 0.98% increase in two experiments; Fig. 5*a*, lanes *3* and *4*). A 70.5 ± 1.5% decrease in two experiments in cyclin A–associated kinase activity was also observed in stable transfectants upon maximal induction of β_{1C} expression at 72 hours (Fig. 5*c*, lane *2*), whereas no effect was seen in mock-transfected cells (Fig. 5*c*, lane *4*). A strong inhibition of cyclin A–associated kinase activity was also observed in NRP152-β_{1C} transfectants (data not shown). Immunoblotting analysis of cyclin E, cyclin A, and CDK2 showed that β_{1C} expression had no effect on the levels of these proteins (Fig. 5*b*, *top* and *middle*, *e*; data not shown).

Because cyclin A–associated kinase activity can be inhibited by increased association of $p27^{kip1}$ to cyclin A-CDK complexes, we analyzed the amount of $p27^{kip1}$ associated with these complexes in β_{1C} transfectants. Comparable amounts of cyclin A were immunoprecipitated from total cell lysates of either β_{1C} or mock-stable transfectants cultured in the absence of tetracycline for 48 or 72 hours, and $p27^{kip1}$ association was analyzed by immunoblotting. A substantial increase in the amount of $p27^{kip1}$ associated with cyclin A was found in β_{1C} transfectants (Fig. 5*d*, lanes *1* and *2*) versus mock-transfected cells (Fig. 5 *d*, lanes *3* and *4*) at both 48 and 72 hours (8-fold and 3.6-fold increase, respectively, in the shown experiment; in two additional experiments, which are not shown, the increase at 72 hours was higher than 10-fold). These results suggest that the inhibition of cyclin A–associated kinase activity observed in β_{1C} transfectants is not a consequence of a decrease in cyclin A expression, but is likely a reflection of its increased association with $p27^{kip1}$. Expression of β_{1C} did not affect cell adhesion to ECM proteins, specifically β_1 integrin ligands such as fibronectin and vitronectin, or to integrin-binding antibodies such as TS2/16 (data not shown). These data suggest that β_{1C} could exert its growth-inhibitory effect via an increase of $p27^{kip1}$ and reduction of cyclin A–associated kinase activity, without affecting cell adhesion.

$p27^{kip1}$ antisense oligonucleotides prevent β_{1C} effect on cell proliferation. To evaluate the role of $p27^{kip1}$ in mediating β_{1C} inhibitory effect on cell growth we used $p27^{kip1}$ antisense oligonucleotides to downregulate its expression. Treatment of CHO-β_{1C} transfectants with $p27^{kip1}$ antisense (AS) oligonucleotides resulted in a strong reduction of $p27^{kip1}$ protein expression levels compared with mismatch (MS) oligonucleotide-treated cells (Fig. 6*c*, *top*). In agreement with our previous published data (9), induction of β_{1C} expression in CHO stable transfectants resulted in a strong inhibition of [^{3}H]thymidine incorporation in response to serum, whereas [^{3}H]thymidine incorporation was stimulated 3.7-fold in mock-transfected cells (Fig. 6*a*). $p27^{kip1}$ antisense oligonucleotides, but not mismatch oligonucleotides, significantly restored DNA synthesis (4.2-fold increase in [^{3}H]thymidine incorporation) in CHO-

β_{1C} stable transfectants in response to serum (Fig. 6*b*). These results show that p27[kip1] mediates β_{1C}-dependent growth inhibition.

Forced β_{1C} expression does not affect MAP kinase activation. Synergistic activity of integrins and mitogenic stimuli leads to activation of some members of the MAP kinase family, specifically of ERK-1 and ERK-2 (5, 34). To investigate the ability of β_{1C} to modulate ERK-1 and/or ERK-2 activation, transient CHO cell transfectants expressing β_{1C} or β_{1A} integrins were used. The cells were kept in suspension or seeded on tissue culture dishes coated with TS2/16 or fibronectin, and MAP kinase activation was analyzed either in the absence or in the presence of 1% fetal calf serum (FCS). The phosphorylation state of ERK-1 and ERK-2 was analyzed by mobility shift (Fig. 7*a*) and by *in vitro* kinase assays (Fig. 7*b*). A synergistic activation of ERK-1 and ERK-2 by β_{1C} or β_{1A} engagement and serum was observed when β_{1C} or β_{1A} transfectants were plated on TS2/16 in the presence of 1% FCS (Fig. 7 *a*, lanes *4* and *6*; *b*, lanes *2* and *4*). Activation of ERK-1 and ERK-2 was not observed when β_{1C} or β_{1A} transfectants were plated on TS2/16 in absence of FCS (Fig 7 *a*, lanes *3* and *5*; *b*, lanes *1* and *3*), whereas β_{1C} transfectants plated on fibronectin were able to activate ERK-1 and ERK-2 (Fig. 7 *a*, lane *7*). Cells held in suspension either in the absence or in the presence (Fig. 7 *a*, lanes *1* and *2*, respectively) of 1% FCS showed very low levels of ERK-1 and ERK-2 activation. These data show that β_{1C} engagement does not prevent ERK-1 and ERK-2 activation in response to mitogenic stimuli or to fibronectin.

Discussion

In this report it is shown, first, that *in vivo* expression of the β_{1C} integrin and of the CKI, p27[kip1], correlates in benign and neoplastic prostate epithelial cells; second, that forced expression of β_{1C} *in vitro* is accompanied by increased levels of p27[kip1] and by selective inhibition of cyclin A-dependent, but not of cyclin E-dependent, kinase activity; third, that increased p27[kip1] association with cyclin A is observed as a consequence of β_{1C} expression; and fourth, that p27[kip1] mediates β_{1C}-dependent growth inhibition. This is the first report showing an *in vivo* correlation between integrins and cell-cycle inhibitors in benign and neoplastic prostate tissue; thus, it brings new insights into the molecular mechanisms underlying prostate cancer progression. Furthermore, this study shows a unique mechanism of regulation of cell growth by integrins.

The results highlight the role of β_{1C} as an upstream regulator of p27[kip1]. Low levels of p27[kip1] recently have been shown to predict an increased risk for treatment failure in lymph node–negative prostate cancer patients (13), and the use of p27[kip1] to evaluate response to therapy and differential treatment decisions has been recommended. Because *in vivo* downregulation of β_{1C} is likely to occur at an earlier stage than loss of p27[kip1] in the pathogenesis of prostate cancer we expect β_{1C} to be a sensitive prognostic indicator of potentially high clinical value to predict therapy and patient survival. Further studies to investigate this area of research are in progress. The β_{1A} integrin that is identical to β_{1C}, except for a different carboxy-terminal cytodomain, was neither downregulated in prostatic adenocarcinoma (8), nor did it inhibit prostate cell proliferation (7), thus indicating a specificity of effect of the

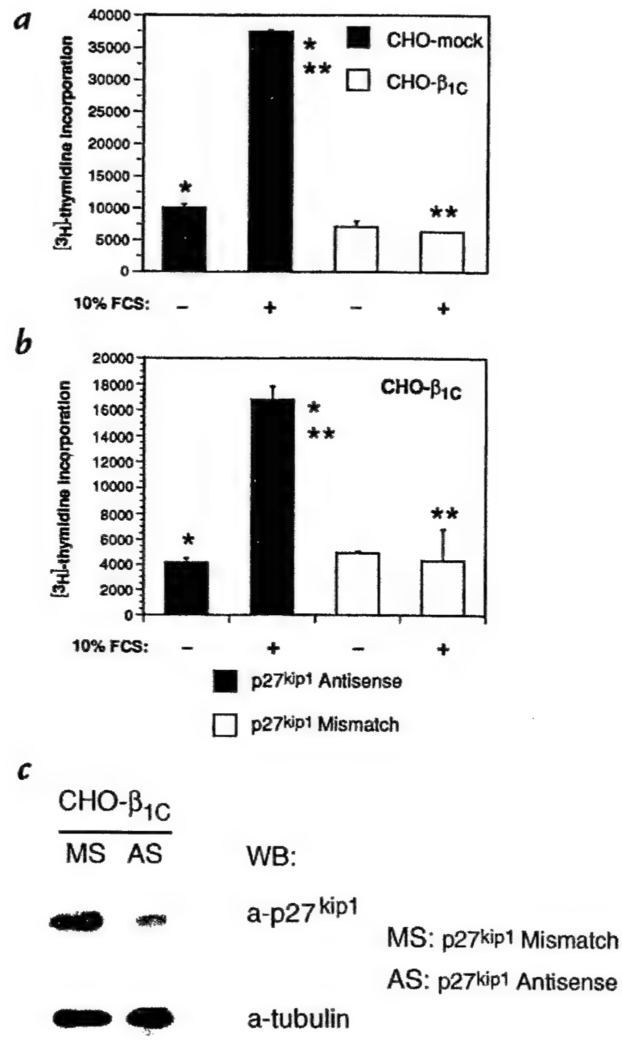

Figure 6

p27[kip1] antisense oligonucleotides prevent β_{1C} inhibitory effect on CHO cell proliferation. The experiments were repeated twice using two different β_{1C} clones with similar results. (***a***) CHO-mock or -β_{1C} stable cell transfectants were cultured for 24 h in tetracycline-free medium, washed three times with PBS, and then incubated for 48 h either in the absence or in the presence of 10% FCS. 1 μCi/well [^{3}H]thymidine was added during the last 3 h of the 48-h culture. (***b***) CHO-β_{1C} stable cell transfectants were transfected using 30 nM of either p27[kip1] antisense or mismatch oligonucleotides. After 24 h, the cells were cultured for additional 48 h either in the absence or in the presence of 10% FCS. 1 μCi/well [^{3}H]thymidine was added during the last 3 h of the 48-h culture. Results are mean ± SEM values of duplicate determinations. Group differences were compared using one-way analysis of variance. In *a*, the differences in proliferation between CHO-mock and CHO-β_{1C} transfectants in the presence of FCS (**) or between CHO-mock cells in the presence of FCS and CHO-mock cells in the absence of FCS (*) are statistically significant ($P < 0.05$). In *b*, the differences in proliferation between antisense- and mismatch-treated transfectants in the presence of FCS (**) or between antisense-treated transfectants in the presence and in the absence of FCS (*) are statistically significant ($P < 0.05$). Consistent results were obtained in a separate experiment where triplicate observations were performed. (***c***) CHO-β_{1C} cells were transfected using 30 nM of either p27[kip1] antisense (*AS*) or mismatch (*MS*) oligonucleotides in the absence of tetracycline. After 24 h, the cells were lysed and p27[kip1] expression levels were evaluated by immunoblotting using 0.8 μg/ml MAB to p27[kip1] (*top*). Control for protein loading was provided by MAB to tubulin (*bottom*). Proteins were viewed by ECL

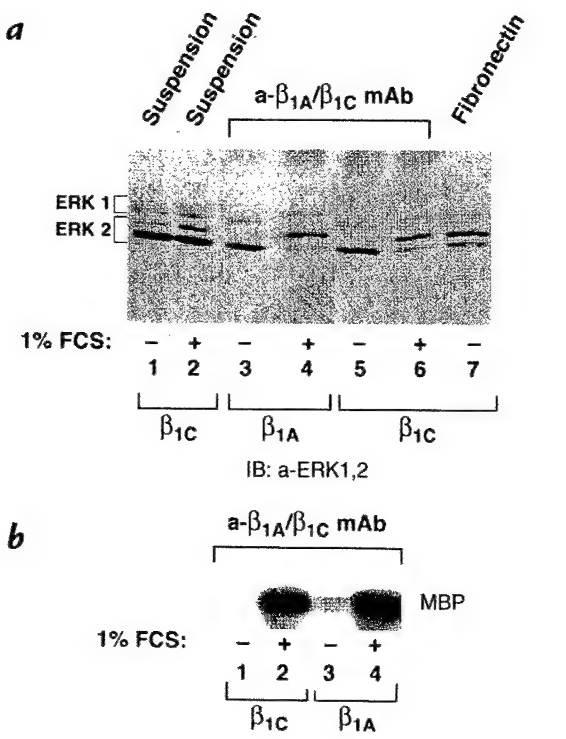

Figure 7

Synergistic activation of ERK-1 and ERK-2 by β_{1C} engagement and serum stimulation. (***a*** and ***b***) Serum-starved CHO cells were transiently transfected using pBJ1-β_{1C} or pBJ1-β_{1A}. The cells were kept in suspension or seeded on tissue culture dishes coated with TS2/16 (*a-β_{1A}/β_{1C} MAB*) or fibronectin for 10 min at 37°C, and MAP kinase activation was analyzed either in the absence or in the presence of 1% FCS. The phosphorylation state of both ERK-1 and ERK-2 was examined by mobility shift assay (*a*) and by *in vitro* kinase assay (*b*). In *a*, detergent cell extracts were analyzed by immunoblotting using 0.1 μg/ml affinity-purified antibody to ERK-1 and ERK-2. Proteins were viewed by ECL. The positions of nonphosphorylated and phosphorylated ERK-1 and ERK-2 are indicated by brackets. In *b*, ERK-1 was immunoprecipitated from total cell lysate using 0.5 μg affinity-purified antibody to ERK-1, and its kinase activity was analyzed using MBP as a substrate. Phosphorylated MBP was seen with autoradiography. The experiments were repeated at least three times with consistent results. In *a*, lanes *1–2*, *5–7*, CHO-β_{1C} cells; lanes *3* and *4*, CHO-β_{1A} cells. In *b*, lanes *1* and *2*, CHO-β_{1C} cells; lanes *3* and *4*, CHO-β_{1A} cells. *MAP*, mitogen-activated protein; *MBP*, myelin basic protein; *ERK*, extracellular signal-regulated kinase.

unique β_{1C} sequence. On the basis of these observations, a potential use of specific β_{1C} sequences can be foreseen to prevent tumor growth *in vivo*.

Incorrect expression of integrins or of their cytodomain in epithelial cells modifies their growth rate *in vivo* and has been shown to generate pathological phenotypes (35–37). Previous data from our laboratory show that β_{1C} integrins in epithelial cells are found in benign and nonproliferative epithelium and are downregulated in prostatic adenocarcinoma (7, 8). Forced expression of the cytoplasmic domain of β_{1C} has a causal role in inhibiting cell proliferation of tumorigenic and highly metastatic prostate cancer cells (7). Thus, because of the ability of β_{1C} to maintain high cellular levels of $p27^{kip1}$ it is expected that deregulation of β_{1C}, and consequently of $p27^{kip1}$ expression in prostate epithelial cells, may be an important step for malignant growth.

The effect of $p27^{kip1}$ antisense oligonucleotides shown in this study confirms the crucial role of $p27^{kip1}$ in modulating β_{1C}-dependent growth inhibition. The pathways controlled by β_{1C} specifically affect cyclin A-CDK activities, but not cyclin E-CDK activities, thus indicating that cyclin A-CDKs and $p27^{kip1}$ are selective downstream targets to this integrin. Although surprising, because of the $p27^{kip1}$ ability to inhibit both cyclin A- and E-CDKs, the findings are suggestive of a unique mechanism regulated by this integrin. The induction of $p27^{kip1}$ preceded the observed decrease in cyclin A–associated kinase activity, thus pointing to $p27^{kip1}$ as the earliest yet identified downstream molecule that links the expression of a specific integrin with cell-cycle regulation. The potential key players and mechanisms necessary to maintain high levels of $p27^{kip1}$ in response to β_{1C} are being studied at this time. Unlike a previous report, where 10T1/2 fibroblasts expressing β_{1C} were used (10), we did not observe changes in cyclin A expression in CHO cells; this finding may be explained by the different nature of the cell types analyzed in the two studies. In a similar manner, it was shown that cyclin D1 expression levels are regulated in an anchorage-dependent manner in 3T3 cells (19, 21), but not in NRK cells (19).

In a previous study, increased levels of another member of the CKI family, $p21^{cip1/waf1}$, which leads rectal carcinoma cells into an irreversible apoptotic pathway were observed in response to β_4 integrin expression (38). Although we have not tested a potential effect of β_{1C} in stimulating apoptosis, this is unlikely to occur because β_{1C} expression appears to affect cell proliferation in a reversible manner. In fact, addition of tetracycline to the growth medium of β_{1C} transfectants did allow successful reculture of all cells, since cells expressing β_{1C} were 100% viable and were all able to reattach to tissue culture plates (Fornaro, M., and Languino, L.R., unpublished results).

The induction of $p27^{kip1}$ observed in β_{1C} transfectants was not accompanied by detectable changes in either cell adhesion to integrin ligands or spreading or focal contact organization; neither was dependent on β_{1C} engagement. Other reports have shown that disruption of cell adhesion to the ECM inhibits cell proliferation by altering levels of cell-cycle molecules, including $p27^{kip1}$ and their activities; in these studies, however, these observations were the result of a complex combination of loss of anchorage, loss of cell spreading, and perturbation of cytoskeletal organization (20–24). Similarly, the decrease in cyclin A–associated kinase activity, shown here, reflects an increased association of $p27^{kip1}$ with cyclin A rather than a transcriptional cyclin A block in response to loss of cell adhesion, as shown previously by several groups (19, 23, 39). Consistent with these observations, adhesion to integrin ligands or adhesion-dependent events, such as cyclin E expression, cyclin E–associated kinase activity, or MAP kinase activation, were not affected by β_{1C} expression. Specifically, the failure of β_{1C} to prevent MAP kinase activation by fibronectin or by synergistic activities of β_{1C} integrin and serum makes unlikely the possibility that upstream mediators of MAP kinase activation, such as FAK, or Shc, or c-Src (4, 40), are inhibited in β_{1C} transfectants; this, however, remains to be proven.

The integrin cytoplasmic domains are key regulators of integrin and cell functions, as well as of intracellular sig-

naling events (41, 42). Recently, β_{1D}, an additional variant form of the β_1 subfamily, has been shown to inhibit cell proliferation (43) and can affect development *in vivo* (44). Because subtle variations in the integrin cytoplasmic domain affect cell proliferation and development, it is conceivable that the expression of these subunits requires a tight transcriptional and translational regulation. Studies in progress in our laboratory on the mechanisms regulating β_{1C} expression in benign and neoplastic prostate will bring new insight into the understanding of the events that contribute to prostate cancer progression.

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Prostatic Carcinoma Cell Migration via $\alpha_v\beta_3$ Integrin Is Modulated by a Focal Adhesion Kinase Pathway[1]

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ABSTRACT

The highly invasive human prostate cancer PC3 cell line was found to express the $\alpha_v\beta_3$ integrin; in contrast, the noninvasive LNCaP prostate cancer cell line did not express $\alpha_v\beta_3$. PC3 cells adhered to and migrated on vitronectin (VN), an $\alpha_v\beta_3$ ligand expressed in mature bone where prostate cancer cells preferentially metastasize. In contrast, LNCaP cells did not adhere to or migrate on VN. Analysis of primary human prostate cancer cells isolated from 16 surgical specimens, showed that these cells expressed $\alpha_v\beta_3$, whereas normal prostate epithelial cells did not. In addition, only primary prostate cancer cells adhered to and migrated on VN. The role of $\alpha_v\beta_3$ in mediating prostate epithelial cell migration was confirmed using LNCaP cell transfectants expressing β_3 (β_3-LNCaP). Exogenous expression of $\alpha_v\beta_3$ induced LNCaP cells to adhere to and migrate on VN. In response to $\alpha_v\beta_3$ engagement, increased tyrosine phosphorylation of focal adhesion kinase (FAK), a signaling molecule activated by integrins and able to modulate cell migration, was detected. Transfection of FAK-related nonkinase, known to compete with FAK for its correct localization and phosphorylation, caused inhibition of β_3-LNCaP cell migration, specifically on VN. These data indicate that *de novo* expression of $\alpha_v\beta_3$ integrin in prostate cancer cells generates a migratory phenotype that is modulated by a FAK signaling pathway. This study points to $\alpha_v\beta_3$ as potential target in prostate cancer cell invasion and metastasis.

INTRODUCTION

Prostatic carcinoma has been estimated to be the second leading cause of death due to cancer among men in the United States (1). Several studies have indicated that prostate cancer cell lines (2–5), as well as human cancer and benign prostatic tissues (6–9), express different members of the integrin family that are known to mediate interactions between cells and ECM[4] proteins. Integrins are heterodimeric cell surface receptors that consist of noncovalently associated α and β subunits; these receptors have been shown to play a role in cell migration, proliferation, and gene transcription and can affect cancer cell invasion and growth (10–15). Alterations of integrin expression in cancer cells correlate with tumor growth and progression, increased invasiveness, and metastatic potential (16).

Integrins provide a direct link between ECM and cytoskeleton, thus, controlling cell motility and, therefore, cancer cell invasion. The $\alpha_v\beta_3$ integrin, specifically, mediates adhesion and migration of several cell types on VN-coated substrates, although its stimulation can result in invasion through basement membrane matrices (17–19). Several receptors for VN have been described; specifically, in epithelial cells, $\alpha_v\beta_5$ integrin can replace the function of $\alpha_v\beta_3$ (20–23). Signaling from the $\alpha_v\beta_3$ can be synergized by growth factor receptors (24, 25).

Several signaling molecules, specifically FAK, Cas, and members of the MAP kinase family, play a role in modulating integrin-mediated cell migration. FAK is a nonreceptor tyrosine kinase localized in focal contacts that becomes tyrosine phosphorylated and subsequently activated on integrin-mediated cell adhesion to several matrix proteins, including VN (26, 27). FAK has been shown *in vitro* to bind β integrin cytoplasmic domain mimetic peptides (28). Domains within the amino- and COOH-terminal regions of the β_3 integrin cytoplasmic tail, including the highly conserved NPXY motif, are required for stimulation of FAK tyrosine phosphorylation (29). In all instances, with the exception of a Cas-binding mutant (FAK P712/715A), FAK is tyrosine phosphorylated in migratory cells (30–34), although in some cell types, FAK tyrosine phosphorylation correlates with reduced migration (35). The COOH-terminal domain of FAK or pp41/43FRNK contains binding sites for a number of signaling molecules, including Cas, as well as a focal adhesion targeting sequence that is sufficient to recruit FAK into focal contacts (36). FRNK acts as a negative regulator of FAK and has been shown, when overexpressed, to prevent tyrosine phosphorylation of FAK and paxillin (37). FRNK has also been shown to delay formation of focal adhesions and cell spreading on FN in chicken embryo cells (37), as well as motility and proliferation of human endothelial cells (32). Cas is a cytoplasmic protein that does not have an intrinsic catalytic activity and that, in response to integrin-mediated cell adhesion, becomes tyrosine phosphorylated and serves as a docking protein for downstream signaling molecules, including FAK (38–40). Recently, increased tyrosine phosphorylation of Cas has been shown to correlate with increased integrin-mediated CHO or COS cell migration (34, 41). Integrin engagement has also been shown to stimulate activation of two members of the MAP kinase family, extracellular-regulated kinase-1 and -2 (14), which are involved in Ras-mediated control of gene expression in response to extracellular stimuli. Additionally, integrin-mediated cell migration of FG pancreatic carcinoma cells, macrophages expressing $\alpha_{6A}\beta_1$, and human umbilical vein endothelial cells has been shown to occur via a mechanism that requires activation of the MAP kinase signaling cascade (42, 43).

In this study, we show that highly invasive human prostate cancer PC3 epithelial cells express the $\alpha_v\beta_3$ integrin and migrate on VN, an $\alpha_v\beta_3$ ligand expressed in mature bone where prostate cancer cells preferentially metastasize. In contrast, noninvasive LNCaP cells do not adhere to or migrate on VN. Primary prostate epithelial cells obtained from prostatic adenocarcinoma, but not cells obtained from normal prostate tissue, express the $\alpha_v\beta_3$ integrin, and only cancer cells adhere to and migrate on VN. Forced expression of $\alpha_v\beta_3$ in noninvasive LNCaP cells generates a migratory phenotype on VN-coated substrates, that correlates with a specific increase in tyrosine phosphorylation of FAK. Cotransfection of β_3 and FRNK prevents cell migration on VN, suggesting a dominant role for FAK in this cellular function. These results describe a novel pathway mediated by the $\alpha_v\beta_3$ integrin that regulates migration of human prostate cancer cells and is relevant to the understanding of the mechanisms that control metastatic spread of these cells.

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[4] The abbreviations used are: ECM, extracellular matrix; FAK, focal adhesion kinase; FRNK, FAK-related nonkinase; MAP, mitogen-activated protein; PE, Phycoerythrin; VN, vitronectin; FN, fibronectin; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; ATCC, American Type Culture Collection; Cas, Crk-associated substrate; CK, cytokeratin.

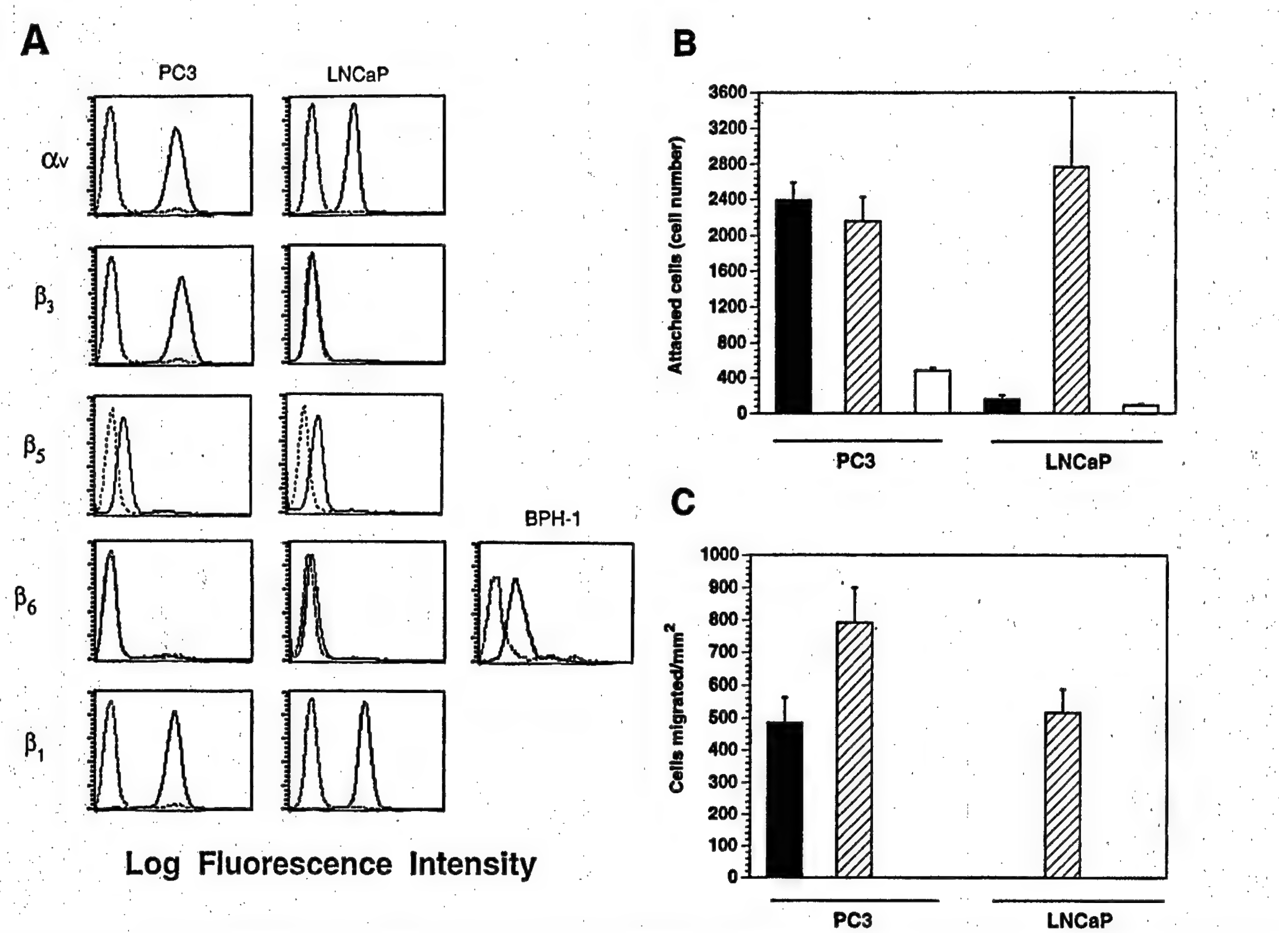

Fig. 1. PC3 and LNCaP cell differential expression of $\alpha_v\beta_3$ integrin and migration on VN. *A*, expression of integrins in PC3 and LNCaP cells by FACS analysis is shown. Monoclonal antibodies to α_v VNR147 (1:500), $\alpha_v\beta_3$ LM609 (1:500), β_5 P1F5 (1:500), β_6 9G6B2 (1:10), and β_1 P4C10 (1:500) were used. BPH-1 cells were used as positive control for the β_6 integrin. *B*, PC3 or LNCaP cells (2.5×10^4) were labeled using [^{51}Cr]sodium chromate and allowed to attach to VN (■, 30 μg/ml), FN (▨, 3 μg/ml), or BSA (□, 10 μg/ml) at 37°C for 2 h. *C*, PC3 or LNCaP cells (8×10^5) were allowed to migrate on VN (5 μg/ml), FN (0.5 μg/ml), or BSA (10 μg/ml) at 37°C for 16 h. BSA was used as negative control. In *B* and *C*, migration and adhesion experiments were repeated at least three times with consistent results. *Bars*, mean ± SE ($n = 3$).

MATERIALS AND METHODS

Cell Lines and Transfections. PC3, LNCaP (ATCC, Rockville, MD), and BPH-1 (provided by Simon W. Hayward, University of California San Francisco, San Francisco, CA; Ref. 44) human prostate epithelial cells were cultured in RPMI 1640 supplemented with 10% (or 2.5% for BPH-1) FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.292 mg/ml l-glutamine (all from Gemini Bio-Products, Inc., Calabasas, CA), 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (Life Technologies, Inc., Gaithersburg, MD). CHO cells (ATCC) were cultured as described (45). To generate stable cell lines, LNCaP cells were transfected with either the full-length human β_{3A} integrin cDNA (designated here as β_3; provided by Tim O'Toole, The Scripps Research Institute, La Jolla, CA) or the full-length human ICAM-1 cDNA in pRc/CMV (designated here as ICAM; provided by Dario C. Altieri, Yale University, New Haven, CT). Chicken FRNK cDNA in the pCMV-c-Myc expression vector, downstream of a c-myc epitope tag, was provided by J. Thomas Parsons and Wen-Cheng Xiong (University of Virginia, Charlottesville, VA). The full-length human β_3 cDNA was excised from CD3a (46) using *XbaI*, subcloned in pRc/CMV mammalian expression vector (Invitrogen, Carlsbad, CA). All transfections were performed using Lipofectin (Life Technologies, Inc.) according to the manufacturer's instructions. pRc/CMV vector alone was used to generate mock-LNCaP-transfected cells. Stably transfected populations were obtained by growing the cells in growth media supplemented with 1 mg/ml geneticin (Life Technologies, Inc.). To obtain a population of cells that uniformly expressed the transfected surface protein, cells were sorted by FACS using either LM609 (1:500; provided by David A. Cheresh, The Scripps Research Institute) against human $\alpha_v\beta_3$ integrin or 2D5 (25 μg/ml) against human ICAM-1. β_3- and FRNK-β_3 (FRNK-β_3-3 and FRNK-β_3-4) LNCaP cell transfectants were also analyzed by immunoblotting for FRNK expression using 0.1 μg/ml C-20, polyclonal antibody to FAK (Santa Cruz Biotechnology, Santa Cruz, CA) that cross-reacts with human and chicken FAK and FRNK. After cell sorting, the stable transfectants were maintained in growth medium supplemented with 0.1 mg/ml geneticin. The levels of expression of β_3-LNCaP in either β_3- or FRNK-β_3 LNCaP cells were comparable, as evaluated by FACS analysis performed using LM609 (data not shown).

Primary Cultures of Epithelial Cells from Human Prostate. Primary cultures of human prostate epithelial cells were prepared as described previously (44). Human prostate tissue specimens were obtained under review board-approved protocols from 16 radical prostatectomies performed for prostatic adenocarcinoma at Yale-New Haven Hospital. Tissue samples of ~0.5 cm^3 were taken from the peripheral zone of the resected prostate in areas grossly suspicious for involvement by carcinoma. The prostate tissue was minced into small pieces ($0.1 \times 0.1 \times 0.1$ cm), ~10% of which was fixed in formalin and embedded in paraffin for histological examination and used as a control. The remaining 90% of the prostate tissue was processed for epithelial cell isolation. Only those samples in which tumor cells represented more than 80% of the total after microscopic examination of the formalin-fixed paraffin-embedded controls were further analyzed. Pathological examination of tissue sections taken from areas immediately adjacent to the 0.5 cm^3 sample obtained for the study further confirmed the presence of carcinoma in all cases. Surgical specimens were collected only from patients that had a localized tumor and lacked metastatic lesions. This study is limited to the use of two normal prostate samples for primary cell isolation, due to obvious difficulties in obtaining fresh normal prostate; autopsy specimens could not be used successfully for primary cell isolation because the specimens were available only several hours after death. For prostate epithelial cell isolation, tissue fragments were dissociated using 200 units/ml Collagenase type I (Sigma Chemical Co.,

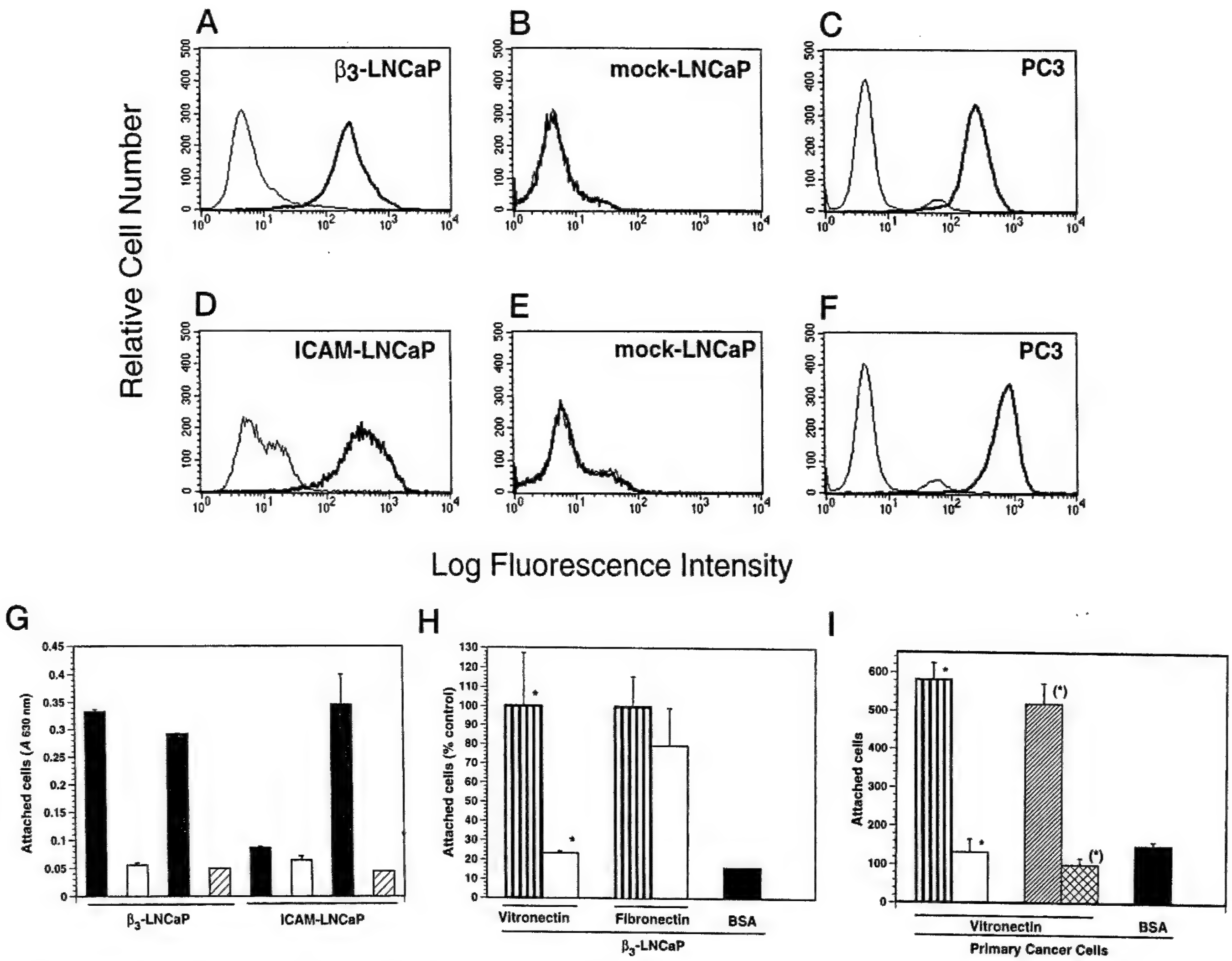

Fig. 2. β_3-LNCaP and primary cancer cells adhere to VN in an $\alpha_v\beta_3$-dependent manner. *A–F*, surface expression of $\alpha_v\beta_3$ is shown by FACS analysis: β_3-LNCaP (*A*), mock-LNCaP (*B* and *E*), PC3 (*C* and *F*), or ICAM-LNCaP (*D*). *A–C*, the *bold line* shows cells labeled using LM609, monoclonal antibody to $\alpha_v\beta_3$, and the *thin line* shows cells labeled using 1C10, negative control monoclonal antibody. *D–F*, the *bold line* shows cells labeled using 2D5, monoclonal antibody to ICAM-1, and the *thin line* shows cells labeled using 14E11, negative control monoclonal antibody. *G–I*, β_3-LNCaP and primary cancer cell adhesion to VN. *G*, β_3-LNCaP or ICAM-LNCaP cells (1×10^5) were allowed to attach to VN (■) or BSA (□; both at 3 μg/ml), L230 (▤), or negative control (▨) hybridoma medium (coating concentrations, 1:500) for 1 h. *H*, β_3-LNCaP cells (1×10^5) were allowed to attach to VN or FN (both at 3 μg/ml) in the presence of LM609 or 1C10 (both at 1:1000) for 105 min. *H*, the results are shown as the percentage of attachment to VN or FN in the presence of the negative control antibody 1C10. The differences between 1C10 and LM609 effects on VN attachment were statistically significant (*, $P < 0.03$; ▥, 1C10; □, LM609). *I*, effect of LM609 on primary prostate cancer cell adhesion to VN. Primary cancer cells (2.5×10^4) labeled by [^{51}Cr]sodium chromate were allowed to attach to VN in the presence of 1C10 (▥), LM609 (□), control IgG (▨), or rabbit antibody (▩) to the VN receptor (all at 1:500), or BSA (▤; 3 μg/ml) for 120 min. The differences in cell adhesion to VN between LM609 and 1C10 or between antibody to the VN receptor and control IgG, indicated by *asterisks*, were statistically significant ($P < 0.05$). In all of the above, consistent results were obtained from at least two different experiments. Representative experiments are shown. *G–I*, *bars* are the mean ± SE ($n = 3$).

St. Louis, MO) and 100 μg/ml DNase I (Sigma Chemical Co.) in PBS at 37°C for 16 h with gentle stirring. The following day, epithelial cells were separated from stromal cells by repeated unit gravity sedimentation. The primary epithelial cells were cultured in RPMI 1640 (Life Technologies, Inc.) supplemented with 2.5% dextran-coated charcoal-stripped heat-inactivated FBS, 1 μg/ml insulin (Sigma Chemical Co.), 10 ng/ml hydrocortisone (Sigma Chemical Co.), 5 μg/ml transferrin (Life Technologies, Inc.), 1 μg/ml sodium folate (Sigma Chemical Co.), 50 ng/ml phosphorylethanolamine (Sigma Chemical Co.), 5 μg/ml ascorbic acid (Sigma Chemical Co.), 5 ng/ml recombinant human epidermal growth factor (R&D Systems, Inc., Minneapolis, MN), and 50 ng/ml cholera toxin (Sigma Chemical Co.) at 37°C in a humidified 7.5% CO_2 incubator. Primary cell characterization was performed using CKs 8 and 18, markers of epithelial cells (47). Indirect immunofluorescence of monolayers of primary cells using monoclonal antibodies to CKs 8 and 18 was performed as follows. Cells isolated by unit gravity sedimentation were seeded onto glass coverslips coated using 3 μg/ml human VN, fixed using acetone (J. T. Baker Inc., Phillipsburg, NJ), blocked using 50 μg/ml BSA (Sigma Chemical Co.) in PBS (pH 7.4; 137 mM NaCl, 2.7 mM KCl, 1 mM Na_2HPO_4, and 1.8 mM KH_2PO_4), incubated with a monoclonal antibody against either human CK 8 (1:300; Boehringer Mannheim, Mannheim, Germany) or CK 18 (1:300; Sigma Chemical Co.), and finally incubated with FITC-conjugated goat antimouse IgG (40 μg/ml; Cappel, Durham, NC) at 37°C for 1 h. Coverslips were washed and mounted on slides with Fluoromount-G (Southern Biotechnology, Birmingham, AL).

Flow Cytometric Analysis. Two-color flow cytometric analysis was performed using the monoclonal antibody against human CK 18 (1:250) and a rabbit serum against the cytoplasmic domain of β_3 integrin (1:200), provided by Erkki Ruoslahti (The Burnham Institute, La Jolla, CA). A monoclonal antibody against a vascular endothelial surface protein, 1C10 (1:250; Life Technologies, Inc.) and nonimmune rabbit serum (1:200) were used as negative controls. Prostate epithelial cell suspensions, either immediately after unit gravity sedimentation or in primary culture, were permeabilized using 0.3% Triton X-100 (Acros, Pittsburgh, PA) for 3 min at room temperature, then blocked using 2% normal horse serum in PBS at 4°C for 15 min. After washing with PBS, the cells were subsequently incubated with antibody to β_3, followed by FITC-conjugated goat antirabbit IgG (40 μg/ml; Jackson, West Grove, PA)

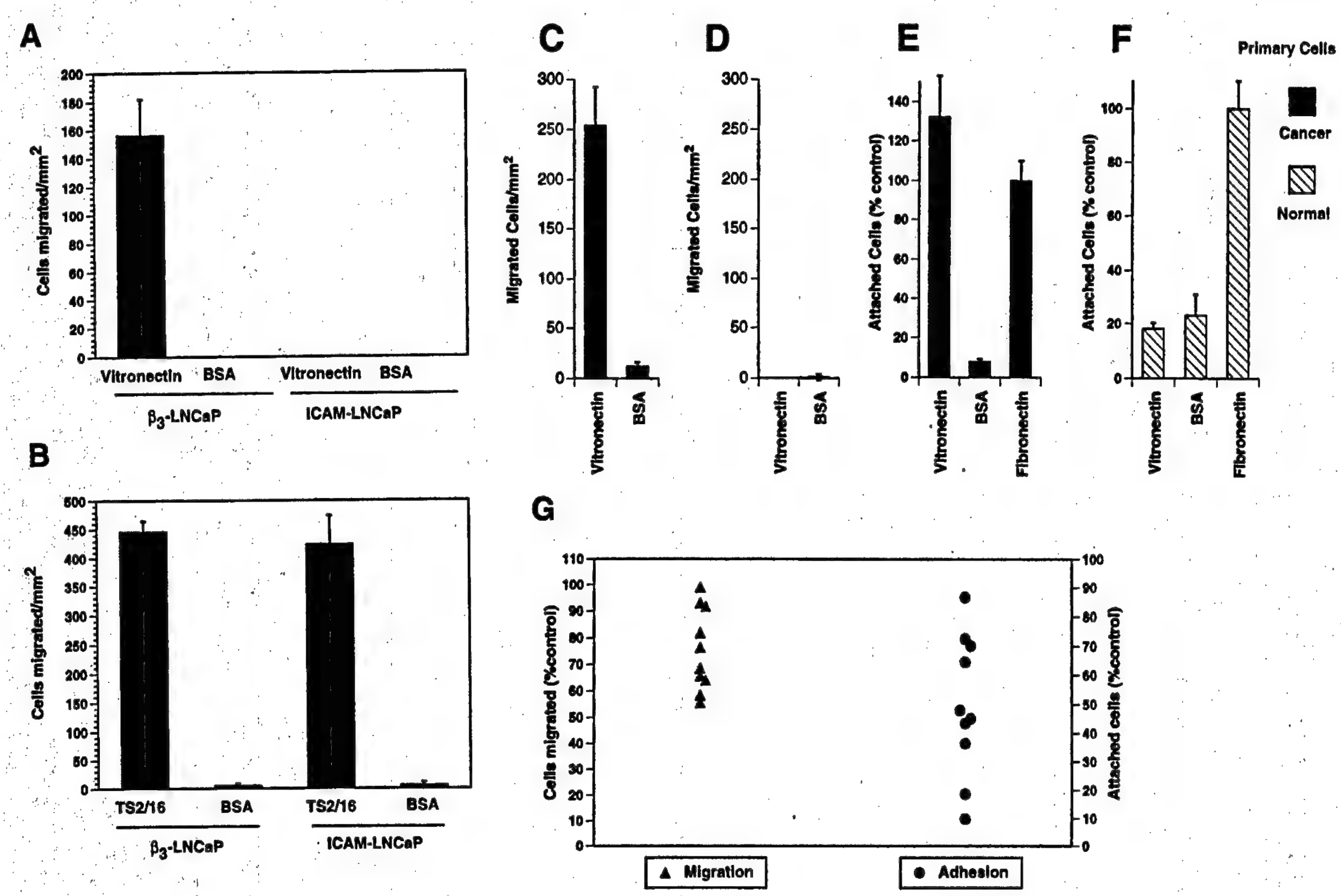

Fig. 3. β_3-LNCaP and primary prostate cancer cells migrate on VN. *A*, β_3-LNCaP or ICAM-LNCaP cells (4×10^5) were allowed to migrate on VN or BSA (both at 3 μg/ml) in each insert at 37°C for 4 h. *B*, β_3-LNCaP or ICAM-LNCaP cells (4×10^5) were allowed to migrate on TS2/16 (coating concentrations, 1:500) or BSA (3 μg/ml) in each insert at 37°C for 4 h. *C–G*, epithelial cells from prostatic adenocarcinoma or normal prostate tissue differentially adhere to and migrate on VN-coated substrates. Epithelial cells (2×10^5) from prostate carcinoma (*C*) or normal (*D*) prostate tissue were allowed to migrate at 37°C for 16 h in Boyden chambers that were coated using VN (3 μg/ml) or BSA (10 μg/ml). The differences between cancer and normal epithelial cell migration on VN were statistically significant ($P < 0.0001$). Epithelial cells (*E*; 2.5×10^4) from prostate carcinoma or normal prostate (*F*) tissue were labeled using [^{51}Cr]sodium chromate and allowed to attach to VN (3 μg/ml), BSA (10 μg/ml), or FN (3 μg/ml) at 37°C for 2 h. *E–G*, the results are expressed as the percentage of control; either migration on or adhesion to FN for each cell population was used as 100% control. *G*, BSA values were subtracted in migration and adhesion assays. The differences between cancer and normal epithelial cell adhesion to VN were statistically significant ($P < 0.05$). Cell migration and adhesion assays were performed using 10 prostatic adenocarcinoma tissue specimens. Each symbol (▲, migrated cells; ●, attached cells) represents one independent case of prostatic adenocarcinoma. *A–F*, *bars* are the mean ± SE ($n = 3$).

and then by antibody to CK 18, followed by PE-coupled goat antimouse IgG (40 μg/ml; DAKO Corp., Carpinteria, CA). The FACS analysis was performed using FACS Vantage (Becton Dickinson, San Jose, CA). One-color FACS analysis was performed using nonpermeabilized epithelial cells with one of the following monoclonal antibodies to human integrins: VNR147 (Life Technologies, Inc.) and L230 (ATCC) to α_v; P1F6 (Life Technologies, Inc.) to $\alpha_v\beta_5$; 9G6B2 (provided by Robert Pytela, University of California San Francisco) to β_6; TS2/16 (ATCC) or P4C10 (Life Technologies, Inc.) to β_1; 1C10; X653, a negative control supernatant; LM609 to $\alpha_v\beta_3$ or 2D5 to ICAM-1; 14E11, a nonbinding antibody used as a negative control; P1E6 (Chemicon, Temecula, CA) to α_2; 9F10 (PharMingen, San Diego, CA) to α_4; P1D6 (Life Technologies, Inc.) to α_5; CLB-701 (Chemicon) to α_6; and P1B5 (provided by Elizabeth Wayner; The Fred Hutchinson Cancer Research Center, Seattle, WA) to α_3. The cells were incubated with goat antimouse FITC-conjugated secondary antibody (40 μg/ml; Cappel) at 4°C for 30 min. FACS analysis and sorting were performed using a FACSort (Becton Dickinson).

Immunoblotting and Immunoprecipitation. Cells from primary cultures were lysed using the following lysis buffer: 50 mM Tris (pH 7.5; American Bioanalytical), 1% NP40 (Calbiochem), 2.5 mg/ml sodium deoxycholate, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM sodium orthovanadate, 20 mM NaF, 0.2 mM EGTA, and 1 mM EDTA (pH 8; all from Sigma Chemical Co.). Antibodies were rabbit sera (1:500), against the cytoplasmic domain of human β_3 or α_v (provided by Erkki Ruoslahti), and nonimmune serum (1:500). Protein concentrations were determined using the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL), and 100 μg of lysate/lane were resolved by 10% SDS-PAGE under reducing conditions. Proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) and immunoblotted. Quantitative analysis was conducted using a computing densitometer (Molecular Dynamics, Sunnyvale, CA). To control for protein loading, membranes were stripped and blotted using rabbit antibodies against SOS-1 (4 μg/ml; Upstate Biotechnology Inc., Lake Placid, NY).

For detection of tyrosine phosphorylated forms of FAK and Cas, cells were detached using 0.05% trypsin and 0.53 mM EDTA, washed once with 0.5 mg/ml soybean trypsin inhibitor and washed twice with RPMI 1640, resuspended in serum-free RPMI 1640, and incubated at 37°C with agitation for 30 min. Cells were then plated on 60-mm dishes coated with either human VN, or human FN, or BSA, as described below, and allowed to adhere at 37°C for 45 min. Cells were lysed in the 1% NP40 lysis buffer described above. The protein concentration of each lysate was determined using BCA protein assay reagent (Pierce Chemical Co.). Precleared lysates were then incubated using either 0.5 μg of C-20, 4 μg of polyclonal antibody to p130Cas (Upstate Biotechnology Inc.), or an equivalent amount of nonimmune rabbit IgG (Sigma Chemical Co.). Western blotting analysis was performed using 1 μg/ml antiphosphotyrosine monoclonal antibody, PY20 (ICN, Costa Mesa, CA), as described previously (48). To detect immunoprecipitated proteins, membranes were stripped and stained using an antibody against FAK, C-20 (0.1 μg/ml), or a monoclonal antibody against Cas (0.25 μg/μl; Transduction Laboratories, Lexington, KY). Experiments were repeated three times.

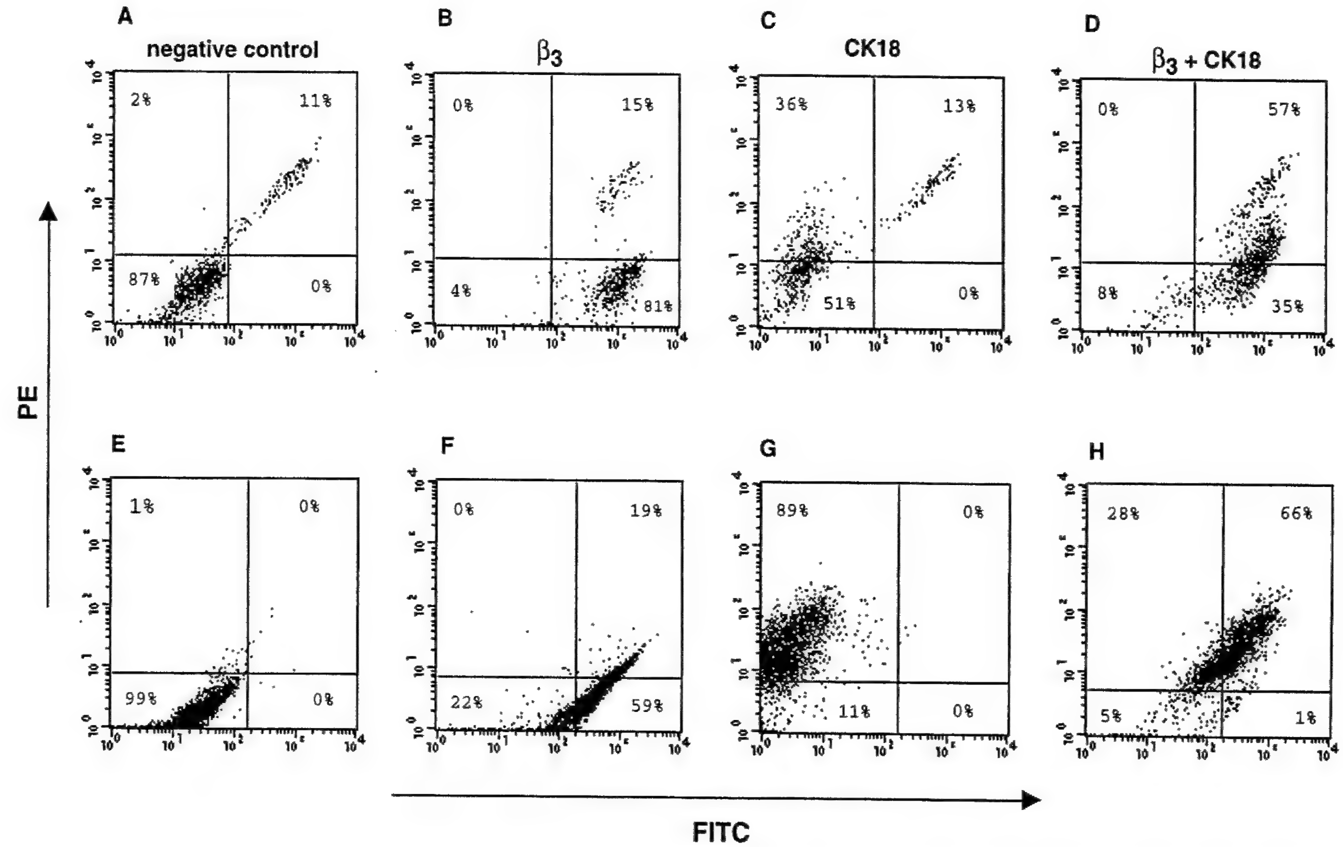

Fig. 4. β_3 integrin and CK 18 coexpression in human prostate cancer cells. Freshly isolated (*A–D*) or primary (*E–H*) cultures of prostate cancer cells were incubated with serum against the cytoplasmic domain of the β_3 integrin and labeled using FITC-conjugated antibody to rabbit IgG (*B* and *F*) or with a monoclonal antibody antihuman CK 18 and labeled using PE-conjugated antibody to murine IgG (*C* and *G*). *D* and *H*, cells were double stained with antibodies to β_3 integrin and to CK 18, followed by FITC- and PE-conjugated antibodies. As negative control, double-staining was performed using nonimmune rabbit serum and 1C10 monoclonal antibody (*A* and *E*), followed by FITC- and PE-conjugated antibodies. Fluorescence intensity is expressed in arbitrary units. The experiment was repeated using either three or two independently obtained prostate cell populations with consistent results. *A–H*, a representative dot plot showing staining for β_3 on the horizontal axis and for CK 18 on the vertical axis is shown; the percentage of cells expressing the single epitope is shown. *D* and *H*, CK 18-positive cells express β_3 (*top right*). *G*, a typical cell population in primary culture was composed of 89% epithelial cells (*top left*).

Adhesion Assay. Adhesion assays were performed as described previously (19). VN and FN were purified as described (49, 50). BSA, L230, or protein-free hybridoma medium (used as a negative control) were from Life Technologies, Inc. For antibody coating, wells were precoated with 10 μg/ml goat antimouse IgG (Cappel). Cell adhesion was quantitated by measuring the absorbance at 630 nm. Inhibition assays were performed by incubating cells in the presence of either LM609 or 1C10 or affinity purified antibodies to the VN receptor (19), or GRGESP or GRGDSPK (1 mg/ml; Life Technologies, Inc). Duplicate observations were performed in each of the above experiments. In some experiments, adhesion was quantitated using cells that had been labeled with [^{51}Cr]sodium chromate (Amersham Corp., Arlington Heights, IL), as described previously (51). Each condition was performed in triplicate.

Migration Assay. Cells (5–8 × 10^5) were resuspended in RPMI 1640 containing 1 mg/ml BSA and 0.5% FBS and plated in transwell migration chambers (12-mm pores from Corning Costar Corporation, Cambridge, MA), as described (19). For antibody coating, the inserts were incubated with RPMI 1640 containing 1 mg/ml BSA and 0.5% FBS at room temperature for 30 min. Cells were allowed to migrate for the indicated times at 37°C in the presence of 5% CO_2. Cells were fixed using 3% paraformaldehyde and subsequently stained with 5 mg/ml crystal violet at room temperature. Cells that had not migrated were removed by wiping the top of the membrane with a cotton swab. The stained cells in 10 randomly chosen fields/filter were counted by microscopic examination. The numbers of migrated cells/mm^2 are shown. In some experiments, cells were incubated on ice for 15 min before plating in the presence of 1:500 dilutions of either LM609 or 1C10, or 1 mg/ml peptides (GRGDSPK or GRGESP).

Statistical Analysis. Statistical analysis was performed using the Student's *t* test or one-way ANOVA, Sigma Stat (Jandel Scientific, San Rafael, CA).

RESULTS

Expression of $\alpha_v\beta_3$ in Prostate Cells Supports Migration on VN. Two prostate epithelial cell lines, PC3 and LNCaP, have been shown to have high and low metastatic potentials, respectively. PC3 cells form i.p. tumors and extravasate from skeletal tissue to form metastatic lesions in nude mice. LNCaP cells do not form i.p. tumors, however, on injection into the medulla of the femur, they do form tumors that are not capable of metastasizing (2, 52). The $\alpha_v\beta_3$ integrin, a receptor for VN and other ligands, was found differentially expressed in these cell lines: specifically, PC3 but not LNCaP cells expressed $\alpha_v\beta_3$ (Fig. 1*A*), as evaluated by monoclonal antibodies to the $\alpha_v\beta_3$ complex (LM609) and to α_v (VNR147). Another VN receptor, $\alpha_v\beta_5$, as well as $\alpha(s)\beta_1$, were expressed at comparable levels in both cell types. A previous study (2) similarly showed lack of expression of $\alpha_v\beta_3$ by LNCaP cells, although α_v-containing complexes were immunoprecipitated by a polyclonal antibody to $\alpha_v\beta_3$. The differential expression of $\alpha_v\beta_3$ in PC3 and LNCaP cells correlated with a different ability of these cells to adhere and migrate on VN (Fig. 1, *B* and *C*). PC3 cells adhered to VN and FN, whereas LNCaP cells adhered only to FN (Fig. 1*B*). Both LNCaP and PC3 cells expressed an endogenous alternative VN receptor on their cell surface, $\alpha_v\beta_5$ (Fig. 1*A*); however, these receptor levels were not able to mediate LNCaP cell adhesion to VN (Fig. 1*B*). PC3 cells migrated on VN and FN, whereas LNCaP cells migrated only on FN (Fig. 1*C*).

To investigate whether the differential expression of $\alpha_v\beta_3$ in PC3

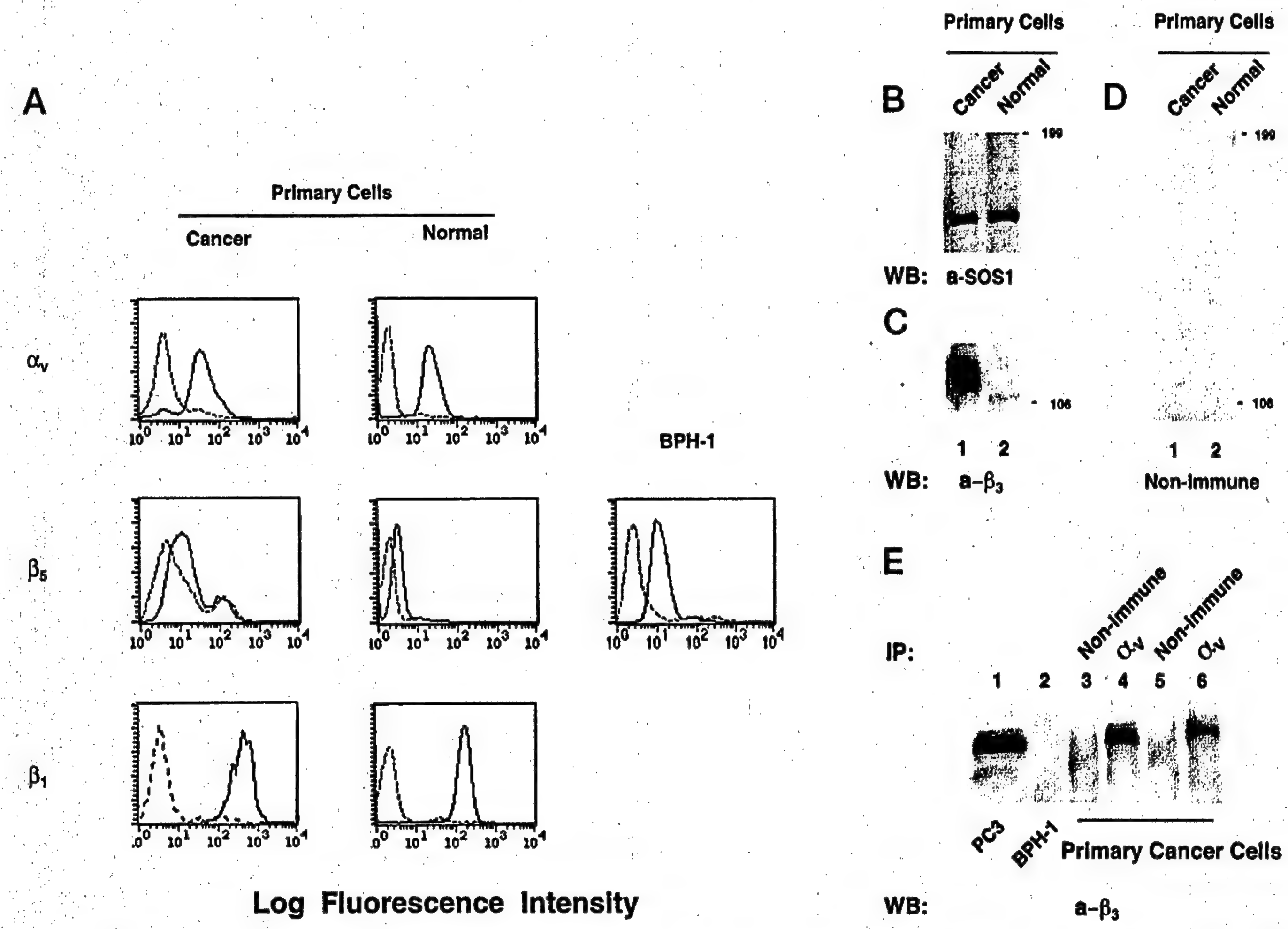

Fig. 5. Differential expression of β_3 integrin in epithelial cells derived from human adenocarcinoma or normal prostate. *A*, FACS analysis of epithelial cells obtained from adenocarcinoma or normal tissue was performed using monoclonal antibodies against human α_v or β_5 or β_1 integrins. BPH-1 cells were used as positive control for the antibody against β_5. Primary epithelial cell lysates from either tumor (*B–D, Lane 1*) or normal (*B–D, Lane 2*) tissue specimens were separated using a 7.5% SDS-PAGE, and immunoblotting was performed using serum against the β_3 integrin (*C*), nonimmune serum (*D*), or an antibody against SOS-1 (*B*); the last was used as control for protein loading. *E*, association of α_v and β_3 integrin subunits was shown by immunoprecipitation using serum against α_v (*Lanes 4* and *6*), followed by immunoblotting using serum against β_3. Nonimmune rabbit serum was used in immunoprecipitations as a negative control (*Lanes 3* and *5*). Two independent primary epithelial cell populations (*Lanes 3* and *4* and *Lanes 5* and *6*, respectively) isolated from tumor specimens are shown. Total lysates from PC3 (*Lane 1*) or BPH-1 (*Lane 2*) cells were used as positive and negative controls for β_3 integrin expression, respectively.

and LNCaP cells could have a causal role in modulating the differing abilities of these two cell types to adhere to and migrate on VN (2, 52), we transfected LNCaP cells using human β_3 integrin cDNA and obtained $\alpha_v\beta_3$ stable transfectants. The transfected β_3 integrin associated with the endogenously expressed α_v, as shown using LM609, an $\alpha_v\beta_3$ complex-specific and function-blocking monoclonal antibody (Fig. 2*A*). As a control, we also generated stable LNCaP cell transfectants that either expressed a distinct surface receptor, the human ICAM-1 (Fig. 2*D*), which is not expressed in LNCaP cells (Fig. 2*E*) and is constitutively expressed in PC3 cells (Fig. 2*F*), or were mock-transfected (Fig. 2*E*). We examined the expression levels of endogenous integrins in mock-transfected LNCaP and in β_3-LNCaP cells to control that potential changes in cell behavior were specifically due to surface expression of $\alpha_v\beta_3$ integrin. We found that exogenous expression of β_3 in LNCaP cells did not significantly alter surface expression levels of the following integrins, which are known to be expressed in epithelial cells (23): α_2, α_3, α_5, α_6, α_v, β_1, and β_5; neither α_4 nor β_6 were expressed on the surface of LNCaP cells (data not shown and Fig. 1*A*).

β_3-LNCaP cells adhered to VN-coated surfaces (Fig. 2*G*) in a concentration-dependent manner (data not shown), whereas ICAM-LNCaP cells did not (Fig. 2*G*). The two cell lines attached comparably well to L230, a monoclonal antibody to α_v (Fig. 2*G*). To confirm that adhesion of the β_3-LNCaP cells to VN occurred in an $\alpha_v\beta_3$-dependent manner, assays were conducted in the presence of LM609. This antibody completely inhibited β_3-LNCaP cell adhesion to VN, but it did not affect attachment to FN (Fig. 2*H*). Similarly, LM609 or an affinity purified antibody to the VN receptor completely inhibited primary prostate cancer cell adhesion to VN (Fig. 2*I*); this inhibitory effect by LM609 was consistently observed using four independent cancer cell populations (data not shown). To further confirm that adhesion to VN occurred via $\alpha_v\beta_3$ and to exclude the role of non-RGD binding receptors for VN, such as the urokinase receptor (53), we tested the ability of a RGD-containing peptide to inhibit attachment. "β_3-LNCaP" cell adhesion to VN was blocked by a RGD peptide, not by a RGE-containing peptide; similarly, inhibition by RGD was consistently observed using three prostate cancer cell populations (data not shown).

To examine whether expression of $\alpha_v\beta_3$ integrin in LNCaP cells correlated with a migratory phenotype, we performed migration assays using a modified Boyden chamber system. Expression of $\alpha_v\beta_3$, but not of ICAM, in LNCaP cells resulted in migration on VN-coated surfaces, confirming that the observed effect was $\alpha_v\beta_3$-dependent

(Fig. 3*A*). Both ICAM-LNCaP and β_3-LNCaP cells migrated equally well on TS2/16, an antibody to human β_1 integrin (Fig. 3*B*).

Differential Migratory Properties of Epithelial Cells from Adenocarcinoma and Normal Tissue. We examined the role of the $\alpha_v\beta_3$ integrin in modulating migration and adhesion of epithelial cells isolated from either prostate carcinoma or from normal prostate tissue. Epithelial cells from prostate carcinoma showed a strong migratory response on VN (Fig. 3*C*) and FN (not shown), whereas epithelial cells from normal prostate tissue did not (Fig. 3*D*). Two cell populations obtained from independent normal tissue specimens showed similar results (Fig. 3*D* and data not shown). The differences in cell migration and adhesion on VN between epithelial cells from prostatic adenocarcinoma and normal tissue were statistically significant ($P < 0.0001$ and $P < 0.05$, respectively). Epithelial cells from prostate carcinoma strongly adhered to VN and FN (Fig. 3*E*), whereas epithelial cells from normal prostate tissue adhered only to FN (Fig. 3*F*). All epithelial cell populations adhered and migrated equally well on FN (data not shown); therefore, FN was used as 100% control in Fig. 3, *E–G*. A migratory response on VN, which was similar or slightly reduced compared with FN-mediated migration, was observed using primary cells obtained from 10 independent prostate carcinoma tissues (Fig. 3*G*); in contrast, the adhesive response to VN of these cell populations was variable. Epithelial cell populations isolated from six additional specimens and analyzed only for their migratory response, consistently migrated on VN (data not shown). Thus, in this regard, PC3 and LNCaP cells seem to have a phenotype similar to cells derived from cancer and normal tissue specimens, respectively.

Primary cancer cells showed a typical epithelial morphology and were stained by CK 8 and CK18 antibodies (data not shown). These cells expressed high levels of the $\alpha_v\beta_3$ integrin; a striking differential expression in cancer and normal cells (Figs. 4 and 5) was found, whereas subtle or no differences were observed in the expression of α_v, β_5, and β_1 integrins (Fig. 5*A*). To confirm that β_3 expression was not due to a contaminant cell population or to culture conditions, we performed two-color flow cytometric analysis (Fig. 4, *A–H*) that is a more sensitive analysis than immunohistochemical staining. Fig. 4, *C* and *G*, shows that ~36% of the cells directly isolated from the tissue specimen, and ~89% of the cells in primary cultures were epithelial because they expressed CK 18. Fig. 4, *D* and *H*, shows that ~46% (57% minus 11%, due to nonspecific staining) of the cells directly isolated from prostate tissue and 66% of the CK 18-positive primary cancer cells expressed β_3.

Lysates of epithelial cells, isolated from either adenocarcinoma or normal tissue, were analyzed by immunoblotting using an antibody against the β_3 cytoplasmic domain (Fig. 5). The results show that epithelial cells isolated from adenocarcinoma expressed β_3, whereas epithelial cells from normal tissue did not (Fig. 5*C*); equal protein amounts were loaded in both lanes, as evaluated using a control antibody to SOS-1 (Fig. 5*B*). As expected, the β_3 integrin formed a heterodimer with the α_v subunit as demonstrated by immunoprecipitation using an antibody to the α_v cytoplasmic domain, followed by immunoblotting using an antibody to the β_3 cytoplasmic domain (Fig. 5*E*). PC3 and BPH-1 prostate epithelial cells are shown as positive and negative controls for β_3 expression (Fig. 5*E*). The β_5, α_v, and β_1 integrin subunits showed a similar expression pattern in cells isolated from either adenocarcinoma or normal tissues (Fig. 5*A*); specifically, the $\alpha_v\beta_5$ integrin, an alternative VN receptor, was found to be poorly expressed. In conclusion, epithelial cells, isolated from fresh adenocarcinoma tissues, express β_3 integrin, and this is not a consequence of *de novo* synthesis of the $\alpha_v\beta_3$ subunit due to cell culture conditions. On the basis of these results, we focused on studying the role of the $\alpha_v\beta_3$ integrin in prostate cancer cell adhesion and migration.

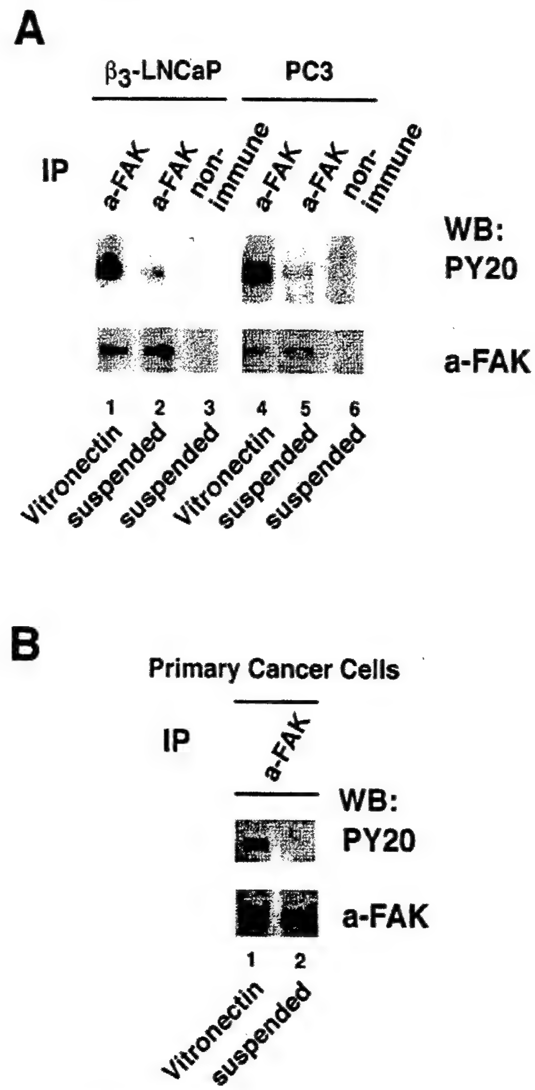

Fig. 6. $\alpha_v\beta_3$ engagement by VN stimulates FAK phosphorylation. *A*, β_3-LNCaP or PC3 cells were plated on VN (*Lanes 1* and *4*; 3 μg/ml) or held in suspension (*Lanes 2, 3, 5,* and *6*) for 45 min and lysed; β_3-LNCaP (500 μg) or 200 μg PC3 lysate/immunoprecipitation were used. *B*, primary epithelial cells from prostatic adenocarcinoma were plated on VN (*Lane 1*; 3 μg/ml) or held in suspension (*Lane 2*) for 70 min and lysed; lysate (300 μg)/immunoprecipitation were used. *A* and *B*, anti-FAK immunoprecipitates were separated by 7.5% SDS-PAGE under reducing conditions and immunoblotted using PY20, antiphosphotyrosine monoclonal (*top*), and C-20, anti-FAK polyclonal antibody (*bottom*).

Engagement of $\alpha_v\beta_3$ in LNCaP Cells by VN Increases FAK Phosphorylation. We examined the phosphorylation state of FAK in β_3-LNCaP, PC3, and primary epithelial cells on $\alpha_v\beta_3$ integrin engagement by VN. It has been reported previously that LNCaP cells, harvested from their own ECM, have a reduced tyrosine phosphorylation of FAK compared with PC3 cells (54, 55). In agreement with this finding, we observed a very low level of FAK tyrosine phosphorylation in LNCaP cells on engagement of $\alpha_5\beta_1$ (data not shown). Fig. 6*A* shows a 7.3-fold increase in FAK tyrosine phosphorylation in β_3-LNCaP cells plated on VN (Fig. 6*A*, *Lane 1*) compared with cells held in suspension (Fig. 6*A*, *Lane 2*); five experiments were performed, and an average of 6.5-fold increase was observed. FAK tyrosine phosphorylation was also increased in PC3 cells plated on VN (Fig. 6*A*, *Lane 4*) compared with cells held in suspension (Fig. 6*A*, *Lane 5*). In primary cells, FAK phosphorylation increased in response to adhesion to VN (Fig. 6*B*, *Lane 1*) compared with cells held in suspension (*Lane 2*). Thus, FAK tyrosine phosphorylation is stimu-

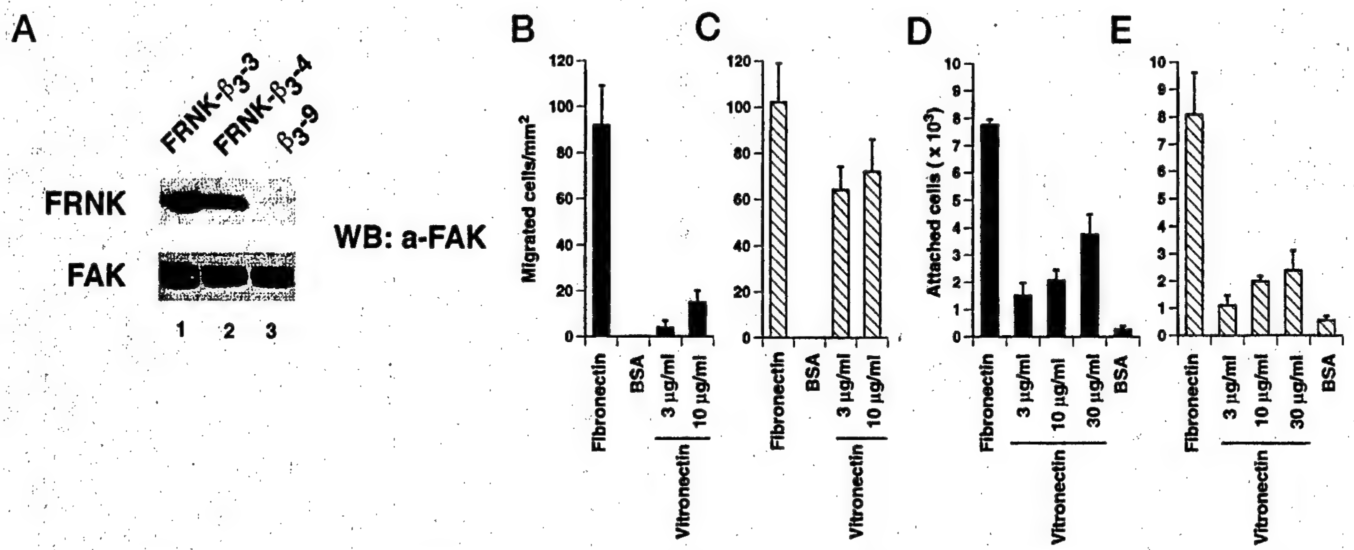

Fig. 7. FRNK expression inhibits β_3-LNCaP cell migration on VN. *A*, LNCaP cells cotransfected using β_3 and FRNK cDNAs are designated FRNK-β_3-3, FRNK-β_3-4, or β_3-9. Each cell lysate (30 μg) was separated using a 7.5% SDS-PAGE and analyzed by immunoblotting using C-20, antibody against FAK (*bottom*), which also recognizes FRNK (*top*). Only FRNK-β_3-3 and FRNK-β_3-4 transfectants expressed detectable levels of FRNK. Computing densitometric analysis showed that FRNK-β_3-3 had >2-fold expression of FRNK compared with FRNK-β_3-4, whereas β_3-9 did not express it. FRNK-β_3-3, FRNK-β_3-4, or β_3-9 expressed comparable levels of endogenous FAK (1.08, 1.00, and 0.94, respectively). *B* and *C*, LNCaP cells (4×10^5) expressing β_3 and FRNK (■), or expressing β_3 but not FRNK (▨), were allowed to migrate on VN (3 and 10 μg/ml), FN (3 μg/ml), or BSA (3 μg/ml) at 37°C for 4 h. *D* and *E*, FRNK-β_3-3 or β_3-9 cells (2.5×10^4)/well were allowed to attach to VN (3, 10, and 30 μg/ml), FN (3 μg/ml), or BSA (3 μg/ml) at 37°C for 2 h. *B–E*, *bars* are the mean ± SE (n = 3).

lated by engagement of $\alpha_v\beta_3$ integrin in human prostate cancer cells. Conversely, Cas tyrosine phosphorylation was not increased in β_3-LNCaP or PC3 cells plated on VN (data not shown) compared with cells held in suspension. CHO cells plated on FN served as a positive control to show that Cas phosphorylation could be detected using the same experimental conditions (data not shown).

Modulation of LNCaP Cell Migration on VN by the FAK-Pathway. To investigate whether FAK signaling would have a causal role in prostate epithelial cell migration, we cotransfected LNCaP cells using FRNK and β_3 cDNAs. LNCaP that, in addition to β_3, expressed FRNK (FRNK-β_3-3 and FRNK-β_3-4; Fig. 7*A* and data not shown) or failed to express FRNK (β_3-9; Fig. 7*A* and data not shown) were analyzed in cell adhesion and cell migration assays (Fig. 7, *B–E*). FRNK expression did not alter LNCaP cell transfectant adhesion to VN (Fig. 7*D*), whereas it did inhibit cell migration on VN (Fig. 7*B*). The effect seemed to be specific for VN because cell motility on FN remained unaffected (Fig. 7, *B* and *C*). Therefore, we conclude that engagement of $\alpha_v\beta_3$ integrin by VN in β_3-LNCaP cells is accompanied by a specific tyrosine phosphorylation of FAK and that the FAK signaling pathway plays a causal role in the migration of these cells.

DISCUSSION

In this study, we show that the highly invasive PC3 cells express the $\alpha_v\beta_3$ integrin and migrate on VN. Tumor-derived human prostate epithelial cells isolated from surgical specimens, but not normal cells, also express $\alpha_v\beta_3$ and migrate on VN. Furthermore, we show that forced expression of the $\alpha_v\beta_3$ integrin induces noninvasive prostate LNCaP cells to migrate on VN. Finally, we demonstrate that the FAK-signaling pathway modulates prostate epithelial cell migration on VN.

The $\alpha_v\beta_3$ integrin, although not frequently found in epithelial cells, is very abundant in bone-residing breast cancer metastases and in malignant ovarian carcinomas (56–58); it is also abundant in metastatic melanomas both *in vivo* (59) and *in vitro* (60). Furthermore, expression of $\alpha_v\beta_3$ causes increased *in vivo* tumorigenicity and metastatic potential of human melanoma cells (61) and predicts subsequent metastatic progression in patients with primary cutaneous melanoma (62). Although the involvement of $\alpha_v\beta_3$ in mediating an invasive phenotype of human prostate cancer cells has not been analyzed due to the obvious difficulties in obtaining suitable samples from patients with metastatic prostate cancer, it can be speculated, on the basis of these observations, that the increased $\alpha_v\beta_3$-mediated migration of prostate cancer cells is likely to generate a metastatic phenotype *in vivo*. It should be stressed that a strong correlation between *in vivo* metastatic spread by β_3-melanoma cell transfectants and *in vitro* β_3-mediated melanoma cell migration, as evaluated using Boyden chamber assays, has been shown (63). Further support to the hypothesis that this integrin plays a role in prostate cancer cell metastatic spread derives from the observation that VN, the best characterized ligand of $\alpha_v\beta_3$, is found in mature bone tissue where these cells preferentially metastasize (64, 65). In addition to VN, another bone matrix protein, osteopontin, binds $\alpha_v\beta_3$; however, it should be pointed out that the role of osteopontin seems to be predominantly in regulating prostate epithelial cell proliferation (66).

De novo expression of the $\alpha_v\beta_3$ integrin and its engagement by VN in prostate cancer cells generate a migratory phenotype that correlates with a specific increase in FAK tyrosine phosphorylation. A correlation between FAK tyrosine phosphorylation and metastatic lesions of prostatic adenocarcinoma has been shown (54). Our results strongly suggest for the first time a causal role for FAK-signaling pathways in prostate epithelial cell migration on VN since FRNK, a negative regulator of FAK, blocks migration of these cells (37). Thus, it is conceivable that activation of FAK will modulate *in vivo* migration and invasion of prostate cancer cells via $\alpha_v\beta_3$. The mechanism that allows inhibition of cell migration by FRNK in a substrate-specific manner (*i.e.*, on VN and not on FN) and the potential ability of FRNK to block parallel pathways, remain to be investigated. A specific role for the β_3 cytodomain in FAK phosphorylation and cell migration has been described by two independent studies, showing that the NPXY motif in the β_3 cytodomain is required to support FAK phosphorylation in fibroblasts (29) as well as in melanoma cell migration and metastatic spread *in vivo* (63). It remains to be established whether

prostate cancer cells also use this motif for their VN-mediated cell migration and FAK phosphorylation.

Cas and PI 3-kinase, both of which form complexes with FAK, are believed to act as downstream effectors of FAK and to control cell migration (39, 40, 67). In our system, although FAK is autophosphorylated and is known to phosphorylate Cas (34), the latter did not seem to be involved in $\alpha_v\beta_3$ signaling in LNCaP cells because its tyrosine phosphorylation remains undetectable in response to VN attachment (data not shown). A role for PI 3-kinase, a signaling molecule that has been shown to play a role in integrin-mediated epithelial cell motility (68, 69), as a potential downstream mediator of $\alpha_v\beta_3$ and FAK-activated pathways, remains to be investigated. Other downstream effectors of FAK are members of the MAP kinase family. The role of MAP kinase in prostate cell migration does not seem to be predominant because a specific inhibitor of MEK-1, PD98059 (70), did not affect cell migration of β_3-LNCaP cells on VN, whereas it did inhibit endothelial cell migration, as described previously (43).[5] Similarly, Cary *et al.* (34) have shown that PD98059 had no effect on FAK/Cas-dependent CHO cell migration, indicating a cell type-dependent activity of the MAP kinase pathway on migration.

Studies performed using tissue sections or cell lines have shown changes in integrin expression between cancer and benign prostate epithelial cells; specifically, redistribution of $\alpha_6\beta_1$ (7) as well as β_{1C} down-regulation in prostate cancer tissues have been described (9); furthermore, $\alpha_6\beta_4$ has been shown to be up-regulated in metastatic prostate cancer cell lines (3). For the first time, in this study, an analysis of integrin expression using prostate cells isolated from fresh tissue samples has been performed. The data show that: (*a*) $\alpha_v\beta_3$ is expressed only by tumor-derived primary cells, but not by normal, prostate epithelial cells; and (*b*) the expression of $\alpha_v\beta_3$ is not induced by culture conditions, but is found constitutively in freshly isolated epithelial cells. Although it is conceivable that ultimately the altered cancerous phenotype will be contributed to by several surface receptors, our study provides, for the first time, evidence that the $\alpha_v\beta_3$ integrin is up-regulated in freshly isolated prostate cancer cells and is a predominant player in the control of migration of these cells. LNCaP cells express $\alpha_v\beta_5$, an alternative receptor for VN (20), that can mediate cell migration of epithelial and melanoma cells on growth factor stimulation (71); however, this integrin that binds VN in several cell types (20, 71, 72) does not participate in β_3-LNCaP binding to VN because a complex-specific antibody to $\alpha_v\beta_3$ completely inhibited β_3-LNCaP cell adhesion to VN.

In conclusion, our study suggests that the $\alpha_v\beta_3$ integrin and the signaling molecules downstream to $\alpha_v\beta_3$ are potential targets to prevent prostate cancer invasion and metastatic spread.

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33

INTEGRINS

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Integrins have emerged as modulators of a variety of cellular functions (1). They have been implicated in organ and tissue development, normal and aberrant cellular growth, and modulation of intracellular signal transduction mechanisms (2–5). In this chapter, the structural and functional characteristics of integrins, their ability to control cell functions and signaling, and their expression and potential role in liver development and disease will be discussed.

THE INTEGRIN FAMILY OF ADHESION RECEPTORS

Adhesive contacts between cells and extracellular matrix (ECM) components play a crucial role in organ development, abnormal tissue growth, and tumor progression (see Chapter 32 and website chapters 🖳 W-27 and W-28. These interactions are mediated by *integrins*, the most widely distributed gene superfamily of adhesion receptors, expressed by all mammalian cells (3). Integrins can also mediate cell–cell interactions, although the ability to mediate cell–cell contact is restricted to a few members of the family [$\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$, $\alpha_D\beta_2$, $\alpha_4\beta_1$, and $\alpha_4\beta_7$(3)].

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α and β Subunits

Based on immunochemical and molecular evidence, integrins are structurally organized into heterodimeric transmembrane complexes, variously assembled through the noncovalent association between an α and a β subunit (6). So far, 18 α subunits, 8 β subunits, and 22 complexes have been identified and their expression and function characterized in various cell types. The integrin family is divided into subfamilies that share the β subunit (7). Each β subunit associates with one to eight α subunits and each α can associate with more than one β subunit. Functional specificity is determined by the specific associated subunits and by the cell type that expresses the heterodimeric complex (Table 33.1).

Integrins are expressed as constitutively active or inactive receptors for ECM ligands. Their functional state is cell type-dependent as well as ligand-dependent (8,9). These different functional states might be crucial in modulating integrin-mediated functions *in vivo*.

Integrin Cytoplasmic Domains

Recent experimental evidence obtained with recombinant deletion mutants and chimeric forms of integrin α and β cytoplasmic domains has demonstrated that cytoplasmic tails modulate receptor distribution, receptor surface expression, ligand binding affinity of the extracellular domain, cell adhesion, and cell spreading (6,10). Therefore, structural differences in the primary sequences of the integrin intracellular domains are predicted to determine the

TABLE 33.1. THE INTEGRIN FAMILY

Subunit		Ligand
$\beta_{1(A)}$	α_1	laminin, collagen
	α_2	laminin, collagen
	α_{3A}, α_{3B}	laminin, collagen, fibronectin entactin
	α_4	fibronectin, VCAM1
	α_5	fibronectin, L1
	α_{6A}, α_{6B}, α_{6X1}, α_{6X2}	laminin
	α_{7A}, α_{7B}, α_{7X1}, α_{7X1X2}	laminin
	α_8	fibronectin, tenascin, vitronectin, osteopontin
	α_9	tenascin
	α_v	fibronectin, osteopontin
β_{1B}, β_{1C}, β_{1D}		
β_2	α_L	ICAM1, ICAM2, ICAM3, ICAM4
	α_M	iC3B, fibrinogen, Factor X, ICAM1, ICAM2, ICAM4
	α_X	iC3b, fibrinogen
	α_D	ICAM3
β_{3A}	α_{IIb}, α_{IIbalt}	fibrinogen, fibronectin, von Willebrand factór, vitronectin, thrombospondin, disintegrin, osteopontin
	α_v	vitronectin, fibrinogen, fibronectin, von Willebrand factor, thrombospondin, disintegrin, L1, MMP2, osteopontin
$\beta_{3B,3C}$		
β_{4A}	α_{6A}, α_{6B}	laminin-5
β_{4B}, β_{4C}, β_{4D}		
β_{5A}	α_v	vitronectin, osteopontin
β_{5B}		
β_6	α_v	fibronectin, tenascin
β_7	α_4	fibronectin, VCAM, MAdCAM1
	α_{IEL}	
β_8	α_v	vitronectin, fibronectin, laminin

specificity of a variety of integrin-mediated events. In support of this hypothesis, mutations and deletions in the integrin cytoplasmic domain have been found in the β_3 and β_4 integrin subgroups in, respectively, Glanzmann's thrombasthenia (11) and junctional epidermolysis bullosa (12), thus pointing to the cytoplasmic domain as a key player in determining crucial cellular responses *in vivo*.

Alternatively spliced forms of the α (α_3, α_6, α_7) and β (β_1, β_3, β_4) integrin cytoplasmic domains have been identified (reviewed in refs. 6,10), thus adding further complexity to the regulatory pathways mediated by integrins. It is well established that the cytoplasmic domain of the β_1 subunit is required for integrins to modulate many cellular functions as well as to trigger signaling events which result in protein phosphorylation and interactions with intracellular proteins (10). Four different β_1 isoforms containing alternatively spliced cytoplasmic domains have been identified (β_{1A}, β_{1B}, β_{1C}, and β_{1D}) that differentially affect receptor localization, cell proliferation, cell adhesion and migration, interactions with intracellular proteins and, ultimately, phosphorylation and activation of signaling molecules (10).

The expression of integrin variants is tissue- and cell type-specific (10). Selective expression has been shown for the β_{1C} integrin subunit, an inhibitor of cell proliferation (10), in hematopoietic cells, platelets, activated endothelial cells and epithelial cells of liver, kidney, lung and prostate. The β_{1B} isoform is restricted to skin and liver, while the β_{1D} subunit was detected in striated muscle, where it replaces the common β_{1A} isoform. Similar to β_1 variants, a differential distribution of the variant forms β_{3A}, β_{3B}, α_{3A}, α_{3B}, α_{6A}, α_{6B}, α_{7A}, and α_{7B} in relationship to their wild-type counterparts has been described using protein and mRNA analysis (10). The functional differences described for these variants suggest that modulation of splicing patterns of β_1 mRNA may provide an accessory mechanism to regulate signaling pathways initiated by integrins (13).

INTEGRIN MODULATION OF CELL PROLIFERATION

By interacting with the ECM and, inside the cell, with the cytoskeleton, integrins transfer signals from the extracellular environment to intracellular compartments and control many cellular functions, such as proliferation, migration, differentiation, and gene expression (14–16). These signals

are initiated after integrin engagement with natural ligands or surrogate antibody ligands and include increases in cytosolic free [Ca2+]i, tyrosine phosphorylation, elevation of intracellular pH, and stimulated transcription and translation of immediate and early inflammatory genes (16). Integrins can act synergistically with growth factors in modulating cellular functions (16).

The ability of integrins to modulate cell proliferation has been extensively characterized (14). Several studies reveal that cell adhesion to the ECM is required for cell cycle progression and proliferation in different cell types (14). Cell adhesion and spreading on fibronectin, vitronectin, and collagen activates mitogen-activated protein (MAP) kinase (17,18). Ras-independent and -dependent pathways have been implicated in MAP kinase activation by integrins (13,19,20). Cell adhesion mediated by integrins modulates the cell cycle, whereas detachment from the matrix induces apoptosis and cell cycle arrest (21). Cyclin A expression and cyclin E-dependent kinase activity are induced by cell attachment to the matrix, adding to the evidence that complex pathways of growth control are mediated by integrins and their ligands (14).

Loss of cell anchorage to the ECM upregulates the expression of the cyclin-dependent kinase inhibitors $p27^{kip1}$ and $p21^{cip1/waf1}$, while decreasing the levels of cyclin A (14). Changes in $p27^{kip1}$ occur in response to integrin expression (22). Overall, these studies show that modulation of cell cycle regulators is mediated by adhesion- and spreading-dependent events as well as by integrin expression.

Integrin ligation contributes to the abnormal proliferation of transformed cells (23) and, in the absence of their ligands, integrins block cell proliferation and downregulate *c-fos* and c-jun early genes. The mechanisms of signaling that occur proximal to the membrane are poorly known; integrin clustering or association with members of the transmembrane 4 superfamily may trigger proliferation signals and, consequently, regulate tumor growth (24).

SIGNALING PATHWAYS ACTIVATED BY INTEGRINS

Integrin engagement and consequent cell adhesion and spreading activate a cascade of intracellular signaling events. The best characterized pathways activated by integrins are Focal Adhesion Kinase (FAK), Phosphoinositide 3 (PI 3)-kinase and Ras/MAP kinase pathways (see Chapters 34–36).

Focal Adhesion Kinase Pathway

Integrins activate the tyrosine kinase FAK, which is a substrate for src (reviewed in refs. 25, 26). FAK is a nonreceptor protein tyrosine kinase that colocalizes with integrins at focal contact sites; FAK becomes tyrosine phosphorylated in response to integrin engagement and prevents apoptosis (26). FAK activation is accompanied by anchorage-independent growth and significant tumorigenic potential (15). FAK binds to paxillin, p130cas, phosphatidylinositol-3 kinase, c-src and Grb2; Grb2 forms complexes with SOS that ultimately activate ras (26). FAK is also activated by mitogens such as platelet-derived growth factor, suggesting a role in mediating the synergistic effects of integrins and growth factors (16). Further investigations are necessary to determine whether integrin-ligand interactions act synergistically or independently from growth factor activity.

Phosphoinositide 3-Kinase Pathway

In addition to stimulating FAK, integrins also activate the phosphoinositide 3 (PI 3)-kinase pathway (27) (see Chapter 35). PI 3-kinases are a family of lipid kinases activated by a wide variety of extracellular stimuli. The lipid products of PI 3-kinases, specifically phosphatidylinositol(3,4)biphosphate [PI(3,4)P_2] and (3,4,5)triphosphate [PI(3,4,5)P_3], affect cell proliferation, survival, differentiation, and migration by targeting specific signaling molecules such as the serine/threonine protein kinase B, also known as AKT, and protein kinase C. Integrin-mediated adhesion to the ECM stimulates the production of PI(3,4)P_2 and PI(3,4,5)P_3, the association of the p85 PI 3-kinase subunit with FAK (27), and AKT activation (28). AKT plays an important role in transducing survival signals in response to several growth factors and integrin engagement (28).

Ras/Mitogen-Activated Protein Kinase Pathway

The small GTPase Ras is a critical component of signaling pathways that control cell proliferation, differentiation and survival. The Ras/extracellular signal-regulated kinase 1 and 2 (ERK1 and 2) MAP kinase pathway plays a pivotal role in modulating gene expression and cell cycle progression in response to mitogens (29). Integrin clustering stimulates Ras GTP-loading and activates specific effectors of the Ras/MAP kinase signaling cascade such as Raf-1 and the MAP kinase, MEK (30).

INTEGRINS AND THE LIVER

The regulated expression of integrins in the liver during development and in healthy and diseased adult liver reflects the functions and cell–cell interactions of the different types of liver cells, and may have an important role in tissue remodeling and disease pathogenesis. Integrin expression in the liver has been well described (see Chapter 32 and website chapters W-27 and W-28); future research will focus on functional correlates, combining general knowledge of integrins with an increasing appreciation of the role of the ECM in the liver.

Integrin Expression in the Developing and Adult Liver

The microenvironment of the liver sinusoid is unique, with a matrix unlike that surrounding any other vascular structure in the body. The two major epithelial cell populations in the adult liver, hepatocytes and biliary epithelial cells, have distinctive integrin profiles reflecting the composition of their basement membranes (Fig. 33.1). The biliary epithelium, which has a standard basement membrane composed of laminin, entactin, perlecan, and type IV collagen, expresses a variety of integrins including the β1 integrins α_2, α_3, α_5, α_6, and α_v, as well as α_6/β_4 (31–33). Hepatocytes lack an organized basement membrane, and their integrin profile, unique among epithelial cells, reflects an adaptation to the special microenvironment of the sinusoid (see Chapters 30 and 31 and website chapter 🖳 W-26). The perisinusoidal matrix includes large amounts of tenascin, but no laminin and entactin; hepatocytes lack the laminin receptors $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$, but express the tenascin receptor $\alpha_9\beta_1$ (31). Hepatocytes also express fibronectin on all surfaces, with corresponding expression of the fibronectin receptor $\alpha_5\beta_1$, and they express $\alpha_1\beta_1$, correlating with their unusual expression (for an epithelial cell) of type I collagen (31,32).

The two epithelial cell populations of the liver diverge from a common precursor cell (Fig. 33.1) (see Chapter 2)

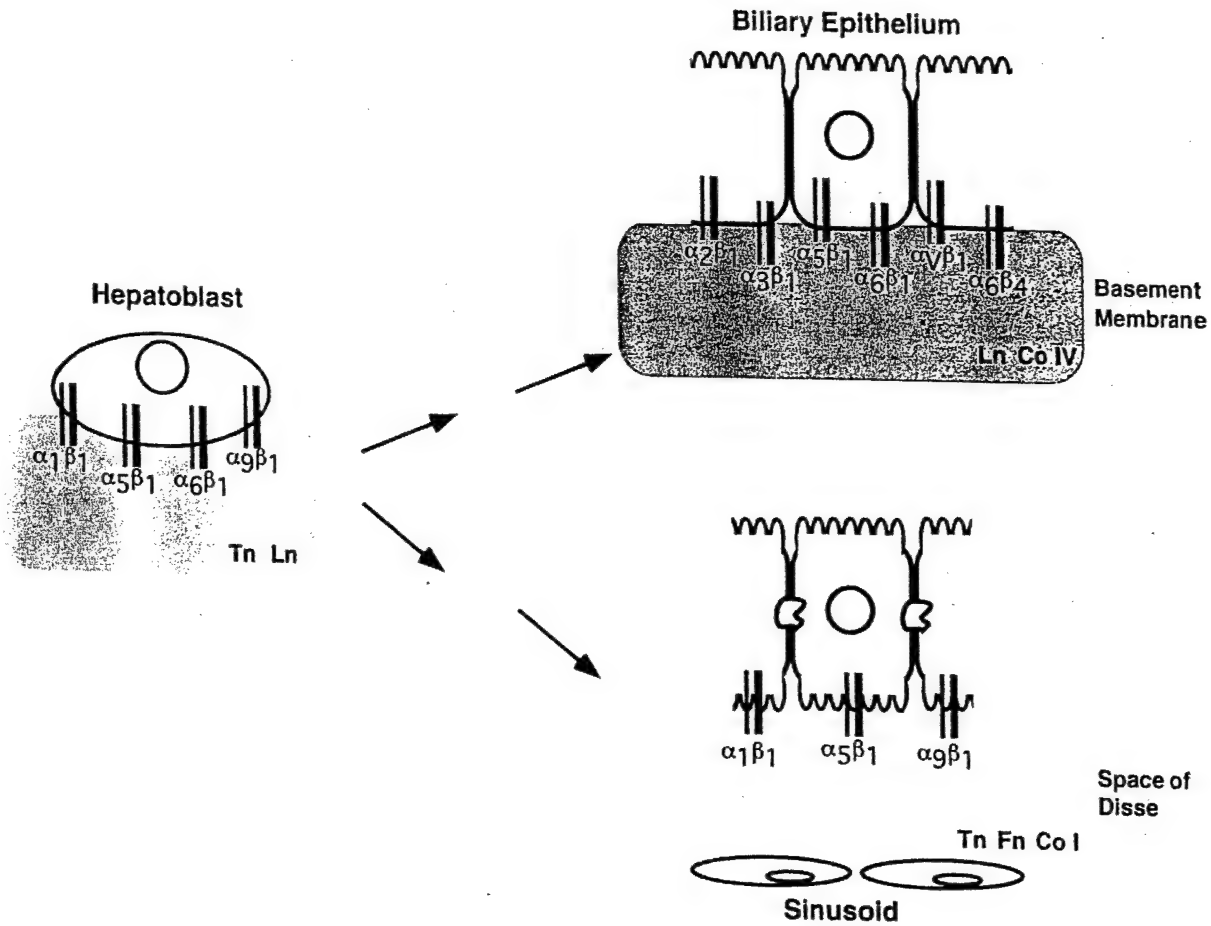

FIGURE 33.1. Integrin expression in the fetal and adult liver. Schematic of a precursor hepatoblast in the 5- to 7-week human liver developing into mature biliary epithelial cells (**top right**) and hepatocytes (**bottom right**) From Couvelard A, Bringuier AF, Dauge MC, et al. Expression of integrins during liver organogenesis in humans. *Hepatology* 1998;27:839–847 and Volpes R, van den Oord JJ, Desmet VJ. Distribution of the VLA family of integrins in normal and pathological human liver tissue. *Gastroenterology* 1991;101:200–206, with permission. Shaded areas represent the basement membrane (biliary epithelium) or its equivalent (perisinusoidal matrix for hepatoblasts and Space of Disse for hepatocytes). The major integrins expressed by the three cell types are shown, as are some of the major matrix components (*Tn*, tenascin; *Ln*, laminin; *Co IV*, type IV collagen; *Co I*, type I collagen; *Fn*, fibronectin). Sinusoidal endothelial cells, shown opposing the hepatocytes, have an integrin expression profile similar to that of hepatocytes. For the sake of clarity, only matrix components that vary among the cell types and are relevant to integrin expression are shown; additionally, details are shown for only one of the hepatocyte sinusoidal surfaces.

early in liver organogenesis (7 to 9 weeks). At the same time, integrin expression on the increasingly distinct cells changes, paralleling changes in the composition of the surrounding ECM (31). Precursor hepatoblasts in the 5 to 7 week human fetal liver express only the β_1 integrins α_1, α_5, α_6, and α_9, similar except for $\alpha_6\beta_1$ to adult hepatocytes. The perisinusoidal matrix, in which the hepatocytes will reside, expresses laminin at 5 to 7 weeks, but has lost it completely by 10 weeks; tenascin increases progressively to reach adult levels by 15 weeks. Primitive hepatocytes, which differentiate from the primitive hepatoblasts between 8 and 30 weeks, gradually lose expression of α_6, consistent with the disappearance of surrounding laminin. By 30 weeks, fetal hepatocytes demonstrate a pattern of integrin expression similar to adult hepatocytes (31).

The biliary epithelium undergoes a marked change in integrin expression beginning at eight weeks, when ductal plate differentiation begins; there is increased α_2, α_3, α_6 and β_4, and decreased α_1, consistent with ECM expression of the developing portal tracts (see Chapter 32 and website chapters 🖳 W-27 and W-28). Laminin and collagen IV expression begins at eight weeks at the point of contact with the ductal plate, and there is a progressive decrease in tenascin expression. The changes in the biliary epithelium are progressive, and there are transitional cells during development that express both $\alpha_1\beta_1$, characteristic of the hepatoblast, and $\alpha_6\beta_1$, characteristic of the mature cells (31). By 12 weeks, the biliary epithelial cells demonstrate a mature pattern of integrin expression. The expression of $\alpha_6\beta_1$ in association with the deposition of laminin on the ductal plate is crucial for bile duct morphogenesis, as these interactions are critical for morphogenesis in other organs. The importance of β_1 integrin subunits in liver development is supported by the finding that mice chimeric for β_1-null cells failed to show colonization of the liver by the knockout cells (34).

Similar interactions may be important in liver regeneration (see Chapter 42 and website chapter 🖳 W-31). After partial hepatectomy, there is rapid and transient upregulation of integrin β_1 and α_V subunits associated with rapid reorganization of other ECM components, suggesting that integrin-mediated processes are important in ECM reorganization and proliferation involved in regeneration (35).

Sinusoidal endothelial cells, which reside in the unique environment of the sinusoid with hepatocytes, have an integrin expression profile similar to hepatocytes, but different from that of most microvascular endothelial cells, including those lining the capillaries of the portal system. Specifically, sinusoidal endothelial cells express high levels of integrins $\alpha_1\beta_1$ and $\alpha_5\beta_1$, consistent with the high levels of collagen and fibronectin in the perisinusoidal space, and $\alpha_9\beta_1$ (32,33,36). They express little or no β_3, β_4, α_2, α_3, or α_6, consistent with the lack of laminin in the perisinusoidal space and with the lack of β_3 ligands such as von Willebrand factor, thrombospondin, and vitronectin.

Integrins in Diseased Liver

The relationship between matrix components and integrin expression persists in inflammatory and cholestatic liver disease, and raises questions about the causal nature and functional relevance of changes in integrin expression. In inflammatory liver diseases, there is an increase in laminin expression, and a corresponding increase in the expression of the laminin receptors $\alpha_3\beta_1$ and $\alpha_6\beta_1$, which are potentially important in liver repair and rearrangement (32). In cholestatic liver disease, hepatocytes can undergo a biliary metaplastic reaction to become more phenotypically similar to biliary epithelial cells, and express increased α_2, α_3, and α_6 (32). Intraductal bipotent stem cells have been described in the human liver (37) and reside within the canals of Hering. Although the integrin expression of these cells has not been reported, the role of integrins in their differentiation is an interesting question for future research.

Integrins in Hepatocellular Carcinoma and Metastases

Tumor cells in hepatocellular carcinoma (HCC) have a markedly different surrounding matrix compared to normal hepatocytes, and a correspondingly different integrin profile that may be functionally relevant. Detailed immunohistologic studies of integrins in normal and cirrhotic liver and in high-grade dysplastic nodules (also referred to as "macroregenerative nodules" and "atypical adenomatous hyperplasia") and HCC demonstrate localization of laminin in the sinusoids of all these except normal liver, with evidence that the laminin is produced by hepatocytes, endothelial cells, and stellate cells (38). There is a corresponding increase in expression of the laminin receptors $\alpha_1\beta_1$ and $\alpha_6\beta_1$. Of these, $\alpha_6\beta_1$, which is not expressed by normal adult hepatocytes, is induced *de novo* during carcinogenesis and correlates best with laminin localization in HCC (38,39). High-grade dysplastic nodules and small HCC have common laminin and $\alpha 6$ subunit expression, lending support to the hypothesis that dysplastic nodules progress to HCC (39). Attachment of various HCC lines to laminin *in vitro* is blocked by antibodies against α_6 and β_1 or by knockouts of $\alpha_6\beta_1$ expression, highlighting the importance of the $\alpha_6\beta_1$ integrin in HCC/laminin attachment (40–42). In these experiments, migration, invasion of basement membrane type matrix, and anchorage-independent growth were also decreased, suggesting that $\alpha_6\beta_1$ has an additional role in maintaining the transformed phenotype of at least some HCC. There is also a focal decrease in the fibronectin receptor subunit α_5 in high-grade dysplastic nodules and HCC, and it has been postulated that this integrin has a role in HCC invasion due to loss of cell–cell and cell–matrix contacts (39). Intrahepatic invasion of human HCC in SCID mice was blocked by antibodies against β_1 and α_5 integrins, supporting this hypothesis (43).

Integrin expression mediates *in vivo* metastasis establishment and growth in the liver and other organs (7). Cell invasion mediated by integrins, a crucial step in *in vivo* metastasis establishment and growth, is supported *in vitro* by signaling molecules FAK, PI 3-kinase, and members of the MAP kinase family. Thus, it is predicted that study of these signaling pathways will contribute to understanding of mechanisms that support metastasis growth *in vivo* in liver and other organs.

Integrins in the Fibrotic Liver

Fibrosis is marked by changes in the integrin expression of hepatocytes and sinusoidal endothelial cells which mimic those seen in HCC, and which reflect alterations in the composition of the perisinusoidal ECM (36). The major change is expression of laminin in the sinusoidal space, where it is normally absent. Correspondingly, laminin receptors on hepatocytes and sinusoidal endothelial cells are upregulated. In hepatocytes, the laminin receptors $\alpha_1\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$ are increased, as well as receptors for collagen and fibronectin ($\alpha_2\beta_1$ and $\alpha_5\beta_1$, respectively). Sinusoidal endothelial cells demonstrate similar increases in these integrins, but also broadly upregulate other integrins including those for $\alpha_3\beta_1$ and $\alpha_4\beta_1$. Of note, these changes occur very early in fibrosis, before the onset of cirrhosis, and may not only represent adaptation to changes in the ECM but also play a causal role in the initiation of fibrosis (36).

Hepatic stellate cells (HSC) and myofibroblasts are the primary matrix-producing cells in the diseased liver, although little is known about their expression of integrins or its relevance to fibrosis (see Chapter 31). In particular, details of stellate cell activation to the fibrogenic phenotype are not known; it is likely that matrix interactions play a major role in initiating this activation.

Stellate cells express integrins $\alpha_1\beta_1$, $\alpha_v\beta_1$, $\alpha_8\beta_1$, and $\alpha_6\beta_4$, consistent with their binding of collagens, fibronectin, and laminin (44,45). $\alpha_2\beta_1$ expression, which occurs in cells *in vitro*, is likely to be an artifact of culture (46). HSC in culture activate if grown on uncoated plastic, but remain quiescent if grown on the basement membrane-like substrate Matrigel; growth on laminin, type IV collagen, and heparan sulfate proteoglycans does not have the same result (47). Interestingly, growing culture-activated cells on Matrigel results in their reversion to the nonfibrogenic, quiescent state (48), suggesting that matrix interactions not only play a role in initiating activation, but also play a role in maintaining the activated state. It may be relevant that integrins are upregulated by the cytokine transforming growth factor (TGF)-β, which is produced in autocrine fashion by activated HSC and is the most potent fibrogenic cytokine in these cells (49). Additionally, TGF-β induces production of the (EIIIA) splice variant of fibronectin by sinusoidal endothelial cells, which contributes to HSC activation (50).

Integrins are also likely to be important in other aspects of HSC function. Integrin $\alpha_1\beta_1$ mediates adhesion to collagen, and may regulate contractility of HSC, potentially related to the development of portal hypertension in cirrhosis (see Chapter 47). Antibodies to this integrin inhibit HSC contraction, although it is expressed constitutively, not just in activated and contractile cells, and there are likely to be other important factors (46). There is a dramatic increase in fibronectin production in the diseased liver; soluble RGD peptides result in decreased production of type I collagen by HSC in culture (by increasing MMP-1 activity) and result overall in decreased activation of these cells through the inhibition of FAK tyrosine phosphorylation, implicating FAK in the cytoskeletal reorganization of HSC as they activate (51,52). Integrins may also be involved in initiation and perpetuation of the inflammatory phase of liver injury: activation of β_1 integrins, by plating on β_1 substrates or by plating on anti-β_1 subunit antibodies, results in increased secretion of monocyte chemotactic protein-1 by HSC (53).

CONCLUSION

Integrins are cell surface receptors for ECM proteins which mediate a variety of functions related to cell proliferation, differentiation, and survival. Although the specific functions of integrins and their ligands in the liver are not well understood, recent publications outlining their expression pave the way for investigations describing integrin isoforms and signaling pathways involved in liver development, malignancy, fibrosis, and differentiation.

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Integrins and prostate cancer metastases

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Key words: integrin, adhesion, migration, survival, proliferation, signaling

Abstract

Integrins have emerged as modulators of a variety of cellular functions. They have been implicated in cell migration, survival, normal and aberrant cellular growth, differentiation, gene expression, and modulation of intracellular signal transduction pathways.

In this review article, the structural and functional characteristics of integrins, their expression and their potential role in prostate cancer metastases will be discussed.

1. Introduction

Integrins are crucial regulators of differentiation, growth, survival, migration and invasion. Thus, they can control the events that characterize the phenotype of a malignant tumor: lack of differentiation, abnormal growth and increased survival, local invasion and infiltration of surrounding normal tissues, and finally, metastatic spread.

In prostate cancer, tumor cells have a markedly different surrounding matrix than normal cells; thus, changes in the integrin profile may be functionally relevant and contribute to metastasis establishment and growth (as discussed below in 4).

The altered integrin and extracellular matrix (ECM) repertoire in metastatic prostate cancer is likely to affect predominantly cell migration. The mechanisms that control cell migration have been shown *in vitro* to be mediated by integrin-activated signaling molecules, such as focal adhesion kinase (FAK), phosphatidylinositol 3-kinase (PI 3-kinase), and members of the extracellular signal-regulated kinase 1 and 2/mitogen-activated protein (ERK1 and 2/MAP) kinase family. Thus, it is predicted that the study of alterations of these signaling pathways controled by integrins will contribute to the understanding of the mechanisms that support metastasis establishment and growth *in vivo* in prostate cancer (as discussed below in 5).

Due to space constraints, studies performed using prostate cancer cell lines *in vitro* will not be discussed in this review.

2. The integrin family of adhesion receptors

Adhesive contacts between cells and ECM components play a crucial role in organ development, abnormal tissue growth, tumor progression and metastatic spread. These interactions are mediated by *integrins*, the most widely distributed gene superfamily of adhesion receptors, expressed by all mammalian cells [1]. Integrins can also mediate cell–cell interactions, although the ability to mediate cell–cell contact is restricted to a few members of the family ($\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$, $\alpha_D\beta_2$, $\alpha_4\beta_1$, and $\alpha_4\beta_7$) [1].

2.1. α and β subunits

Integrins are structurally organized into heterodimeric transmembrane complexes, variously assembled through the non-covalent association between an α and a β subunit [1]. So far, 18 α subunits, 8 β subunits, and 24 complexes have been identified and their expression and function characterized in various cell types. The integrin family is divided into subfamilies that share the β subunit [2]. Each β subunit associates with one to twelve α subunits and each α can associate with more than one β subunit. Functional specificity is determined by the specific associated subunits and by the cell type that expresses the heterodimeric complex (Table 1).

Integrins are expressed as constitutively active or inactive receptors for ECM ligands. Their functional state is cell type-dependent as well as ligand-dependent [3,4]. These different functional states might

Table 1. The integrin family

Subunit		Ligand
$\beta_{1(A)}$	α_1	Laminin, Collagen
	α_2	Laminin, Collagen
	α_{3A}, α_{3B}	Laminin, Collagen, Fibronectin, Entactin
	α_4	Fibronectin, VCAM1
	α_5	Fibronectin, L1
	α_{6A}, α_{6B}, α_{6X1}, α_{6X2}	Laminin
	α_{7A}, α_{7B}, α_{7X1}, α_{7X1X2}	Laminin
	α_8	Fibronectin, Tenascin, Vitronectin, Osteopontin
	α_9	Tenascin
	α_{10}	Collagen
	α_{11}	Collagen
	α_v	Fibronectin, Osteopontin, TGFβ-LAP
β_{1B}, β_{1C}, $\beta_{1C\text{-}2}$, β_{1D}		
β_2	α_L	ICAM1, ICAM2, ICAM3, ICAM4
	α_M	iC3b, Fibrinogen, Factor X, ICAM1, ICAM2, ICAM4
	α_X	iC3b, Fibrinogen
	α_D	ICAM3
β_{3A}	α_{IIb}, α_{IIbalt}	Fibrinogen, Fibronectin, von Willebrand Factor, Vitronectin, Thrombospondin, Disintegrin, Osteopontin
	α_v	Vitronectin, Fibrinogen, Fibronectin, von Willebrand Factor, Thrombospondin, Disintegrin, L1, MMP2, Osteopontin
β_{3B}, β_{3C}		
β_{4A}	α_{6A}, α_{6B}	Laminin-5
β_{4B}, β_{4C}, β_{4D}		
β_{5A}	α_v	Vitronectin, Osteopontin, TGFβ-LAP
β_{5B}		
β_6	α_v	Fibronectin, Tenascin, Vitronectin, TGFβ-LAP
β_7	α_4	Fibronectin, VCAM, MAdCAM1
	α_{IEL}	
β_8	α_v	Vitronectin, Fibronectin, Laminin, TGFβ- LAP

be crucial in modulating integrin-mediated functions *in vivo*.

2.2. *Integrin cytoplasmic domains*

Recent experimental evidence obtained with recombinant deletion mutants and chimeric forms of integrin α and β cytoplasmic domains has demonstrated that cytoplasmic tails modulate receptor distribution, receptor surface expression, ligand binding affinity of the extracellular domain, cell adhesion, and cell spreading [5,6]. Therefore, structural differences in the primary sequences of the integrin intracellular domains are predicted to determine the specificity of a variety of integrin-mediated events. In support of this hypothesis, mutations, and deletions in the integrin cytoplasmic domain have been found in the β_3 and β_4 integrin subgroups in, respectively, Glanzmann's thrombasthenia [7] and junctional epidermolysis bullosa [8], thus pointing to the cytoplasmic domain as a key player in determining crucial cellular responses *in vivo*.

Alternatively spliced forms of the α (α_3, α_6, α_7) and β (β_1, β_3, β_4, β_5) integrin cytoplasmic domains have been identified (for review see [5] and [6]) thus adding further complexity to the regulatory pathways mediated by integrins. It is well established that the cytoplasmic domain of the β_1 subunit is required for integrins to modulate many cellular functions as well as to trigger signaling events which result in protein phosphorylation and interactions with intracellular proteins [6]. Five different β_1 isoforms containing alternatively spliced cytoplasmic domains have been identified (β_{1A}, β_{1B}, β_{1C}, β_{1C-2}, and β_{1D}) and have been shown to differentially affect receptor localization, cell proliferation, cell adhesion and migration, interactions with intracellular proteins and, ultimately, phosphorylation and activation of signaling molecules [6].

The expression of integrin variants is tissue and cell-type specific [6]. A selective expression has been shown for the β_{1C} integrin subunit, an inhibitor of cell proliferation [6], in hematopoietic cells, platelets, activated endothelial cells, and epithelial cells of liver, kidney, lung, breast as well as prostate [6,9–12]. The β_{1B} isoform has been found to be restricted to skin and liver, while the β_{1D} subunit has been detected in striated muscle, where it replaces the common β_{1A} isoform. Similar to β_1 variants, a differential distribution of the variant forms β_{3A}, β_{3B}, α_{3A}, α_{3B}, α_{6A}, α_{6B}, α_{7A}, and α_{7B} in relationship to their wild type counterparts has also been described using protein and mRNA analysis [6]. The functional differences described for these

variants suggest that modulation of splicing patterns of β_1 mRNA may provide an accessory mechanism to regulate signaling pathways initiated by integrins [13–15].

3. Integrin modulation of cellular functions

By interacting with the ECM and, inside the cell, with the cytoskeleton, integrins transfer signals from the extracellular environment to intracellular compartments and control many cellular functions, such as migration, survival, proliferation, differentiation, and gene expression [16–18]. These signals are initiated after integrin engagement with natural ligands or surrogate antibody ligands and include increases in cytosolic free $[Ca^{2+}]_i$, tyrosine phosphorylation, elevation of intracellular pH, and stimulated transcription and translation of immediate and early inflammatory genes [18]. Integrins can act synergistically with growth factors in modulating cellular functions [18]. Overall, the published studies show that such modulation of cellular functions is mediated by adhesion- and spreading-dependent events as well as by integrin expression. A series of excellent reviews are available on these topics [19–22].

It is worth mentioning that $p27^{kip1}$ levels are regulated in response to integrin expression and engagement and are regulated in prostate cancer. Cell adhesion to the ECM is required for cell cycle progression and proliferation in different cell types [16]. Loss of cell anchorage to the ECM has been shown to up-regulate the expression of cyclin kinase inhibitors (CKIs) such as $p27^{kip1}$, while at the same time decreasing the levels of cyclin D1 and A [16]. Engagement of β_1 integrins has been shown to regulate the cell cycle machinery by modulating $p27^{kip1}$ protein levels in either a positive or a negative fashion depending upon the cellular context [9,23,24]. $p27^{kip1}$ is a CKI that controls cell cycle progression by associating with cyclin D-, E-, and A-cdk complexes. $p27^{kip1}$ is highly expressed in non-proliferative, quiescent cells and its levels are increased by growth-inhibitory signals. Furthermore, its forced overexpression is sufficient to inhibit cell proliferation. The pathophysiological relevance of $p27^{kip1}$ regulated expression is suggested by recent studies showing that in prostate cancer, as well as in several types of cancer, loss of $p27^{kip1}$ is an adverse prognostic factor that correlates with poor patient survival [25–29]. Some reports have also shown that low $p27^{kip1}$ expression correlates with lymph node metastasis [30–32]. Previous data from our laboratory have shown that β_{1C} integrin is down-regulated in prostate cancer and that forced expression of β_{1C} *in vitro* is accompanied by an increase in $p27^{kip1}$ levels [9]. Moreover, *in vivo* β_{1C} integrin and $p27^{kip1}$ expressions are concurrently down-regulated in neoplastic prostate epithelial cells, thus describing for the first time, an *in vivo* correlation of expression of integrins and a cell cycle inhibitor [9]. The results highlight the role of β_{1C} as an upstream regulator of $p27^{kip1}$. Since *in vivo* down-regulation of β_{1C} is likely to occur at an earlier stage than $p27^{kip1}$'s loss in the pathogenesis of prostate cancer, we expect β_{1C} to be a sensitive prognostic indicator of potentially high clinical value to predict therapy and patient survival.

4. Integrin expression in prostate cancer

Integrin expression in normal prostate and various prostate cancer specimens has been investigated by several laboratories, most typically by using immunohistochemical techniques (Table 2). With one exception (LM609 antibody that recognizes the $\alpha_v\beta_3$ integrin), the antibodies recognize epitopes on single integrin subunits. In order to extract information regarding the changes in functional integrin heterodimers, the findings must therefore be interpreted based on what is known about the association of integrin subunits.

Dramatic changes seen in integrin levels are those of β_{1C}, β_3, β_4 and a truncated version of α_{IIb}, which lacks the transmembrane and cytoplasmic domains. β_{1C} is expressed in benign glandular epithelial cells, but is markedly down-regulated in adenocarcinomas, regardless of the histological grade [9,12,33,34]. In contrast, β_3 is undetectable in normal prostate, but is expressed in adenocarcinoma and metastatic lesions [35] (Jain, Zheng and Languino, unpublished). β_3 is known to associate with α_v and α_{IIb}. However, even though the levels of α_v have been shown to be decreased in carcinoma compared to normal or benign prostate [36], $\alpha_v\beta_3$ is known to be present on primary prostate adenocarcinoma cells as a functional vitronectin receptor [35]. The truncated α_{IIb} integrin contains a unique sequence at its carboxy terminus, which enabled its detection in adenocarcinoma cells [37]. This epitope was not detected in normal prostate [37]. The integrin studied most intensively is $\alpha_6\beta_4$. β_4, paired with α_6 in hemidesmosomal structures at the interface of normal glandular basal cells and basal lamina, disappears as cells in prostatic

Table 2. Altered expression of integrins in prostate cancer

Integrin	Sample	Method	Altered expression	Reference
β_1	Tissue	IHC	Up-regulated and redistributed with progression	Knox et al. (1994); Murant et al. (1997)
β_{1C}	Tissue	IHC, immunoblot	Down-regulated in adenocarcinoma, expressed in benign epithelium	Fornaro et al. (1996, 1998, 1999); Perlino et al. (2000)
β_3	Freshly isolated cells from tissue and primary cultures, tissue	FACS analysis, immunoblot, IHC	Expressed in adenocarcinoma and metastatic lesions, not in normal cells	Zheng et al. (1999); #Jain et al. unpublished
β_4	Tissue	IHC	Down-regulated in carcinoma	Nagle et al. (1995); Allen et al. (1998); Davis et al. (2001)
α_2	Tissue	IHC	Down-regulated in carcinoma, up-regulated in metastases	Nagle et al. (1994); Bonkhoff et al. (1993)
α_3	Tissue	IHC	Down-regulated in carcinoma	Nagle et al. (1994)
α_4	Tissue	IHC	Down-regulated in carcinoma	Nagle et al. (1994)
α_5	Tissue	IHC	Down-regulated in carcinoma	Nagle et al. (1994)
α_6	Tissue	IHC, TEM	Polarized distribution in benign, less polarized in HGPIN, not polarized in lymph node metastases; hemidesmosomal α6 absent in carcinoma cells; up-regulated in metastases	Bonkhoff et al. (1993); Knox et al. (1994); Nagle et al. (1995)
α_v	Tissue	IHC	Down-regulated in carcinoma	Nagle et al. (1994)
α_{IIb} (truncated)	Tissue	IHC	Expressed in adenocarcinoma, not in normal tissue	Trikha et al. (1998)

IHC: immunohistochemistry; TEM: transmission electron microscopy; FACS: fluorescence activated cell sorting; HGPIN: high grade prostatic intraepithelial neoplasia; #Jain, Zheng and Languino, unpublished results.

intraepithelial neoplasia (PIN) lesions become transformed, and is absent in carcinoma cells [36,38–40]. The expression of α_6, however, is still maintained in prostatic neoplasms, but its distribution becomes more disperse and its density at sites of contact with the basement membrane diminishes with increasing histologic grade [41,42]. Since the only other integrin that α_6 is known to associate with is β_1, this re-distribution of α_6 probably represents the $\alpha_6\beta_1$ integrin. In addition, α_6 is up-regulated in lymph node metastases compared to primary lesions [41]. Most other α integrins, that is, α_2, α_3, α_4, α_5 and, as mentioned above, α_v, have been reported to be down-regulated in adenocarcinoma [36,38]. In one study, increased staining for α_2 was found in lymph node metastases compared to primary lesions [41]. While not distinguishing between the four known different isoforms of β_1, Murant et al. [43] report a slight increase in expression levels of β_1 with increasing Gleason grade. Taken with the reduction of the aforementioned α integrins, this would therefore represent a shift in β_1 heterodimer composition with the progression of prostate cancer. However, it should be noted that some of the α integrin subunits that heterodimerize with β_1, that is, α_1, α_7, α_8, and α_9, have not yet been investigated.

The expression of integrins in normal prostate and prostate cancer has also been investigated at the mRNA level. Indirectly, a massive effort is represented by the Cancer Genome Anatomy Project (CGAP). At the CGAP web site, libraries prepared from many different samples, ranging from normal tissue to metastatic lesions, can be compared. However, not much information regarding integrins is yet available. Even so, integrin protein levels cannot be inferred from mRNA levels. This has been documented in two recent studies. In an investigation of β_1 variant gene expression in normal and neoplastic prostate, Perlino et al. [12] showed that β_{1C} mRNA levels were down-regulated in neoplastic specimens, in agreement with β_{1C} protein levels, but total β_1 mRNA was also down-regulated, in contrast to total β_1 protein levels. Recently, Hao et al. [44] showed that the mRNA of β_4 was at least at the same

level in malignant as compared to normal tissue, which is unexpected given the well-documented decrease in β_4 protein levels. These recent findings indicate that the control of integrin expression in the progression of prostate cancer is complex and deserves further investigations.

5. Signaling pathways activated by integrins: Molecular alterations in prostate cancer

Integrins are likely involved in cancer initiation and/or progression because of their ability not only to mediate interactions with ECM proteins, but also to regulate multiple intracellular signaling molecules that are necessary for cell motility, cell survival and proliferation [1,21]. The mechanisms of signaling that occur proximal to the membrane are poorly known; integrin clustering or association with members of the transmembrane 4 superfamily might be ways to trigger proliferation signals and, consequently, regulate tumor invasion and growth [45].

This section focuses on gene products that have been shown to be involved in signaling events mediated by integrins that affect cell motility and whose expression levels and activity are altered in prostate cancer. The best characterized pathways activated by integrins are the FAK, the PI 3-kinase and the Ras/MAP kinase pathways (Figure 1 and Table 3). Other molecules that are regulated by integrins but have not been implicated in cell migration, such as the tumor suppressors p53 and Rb, cyclins D and A, and the cyclin kinase inhibitor $p21^{cip1}$ have been shown to be frequently mutated and/or overexpressed in prostate cancer. An overview of the most frequent alterations of these molecules in prostate cancer can be found in recent excellent reviews [46,47] and elsewhere in this issue.

5.1. FAK

FAK is a non-receptor protein tyrosine kinase that has been shown to co-localize with integrins at focal contact sites [48]. FAK becomes tyrosine phosphorylated in response to integrin engagement and other stimuli [48–50]. FAK inhibition induces apoptosis and overexpression of FAK prevents apoptosis induced either in absence of ECM survival signals or in response to other

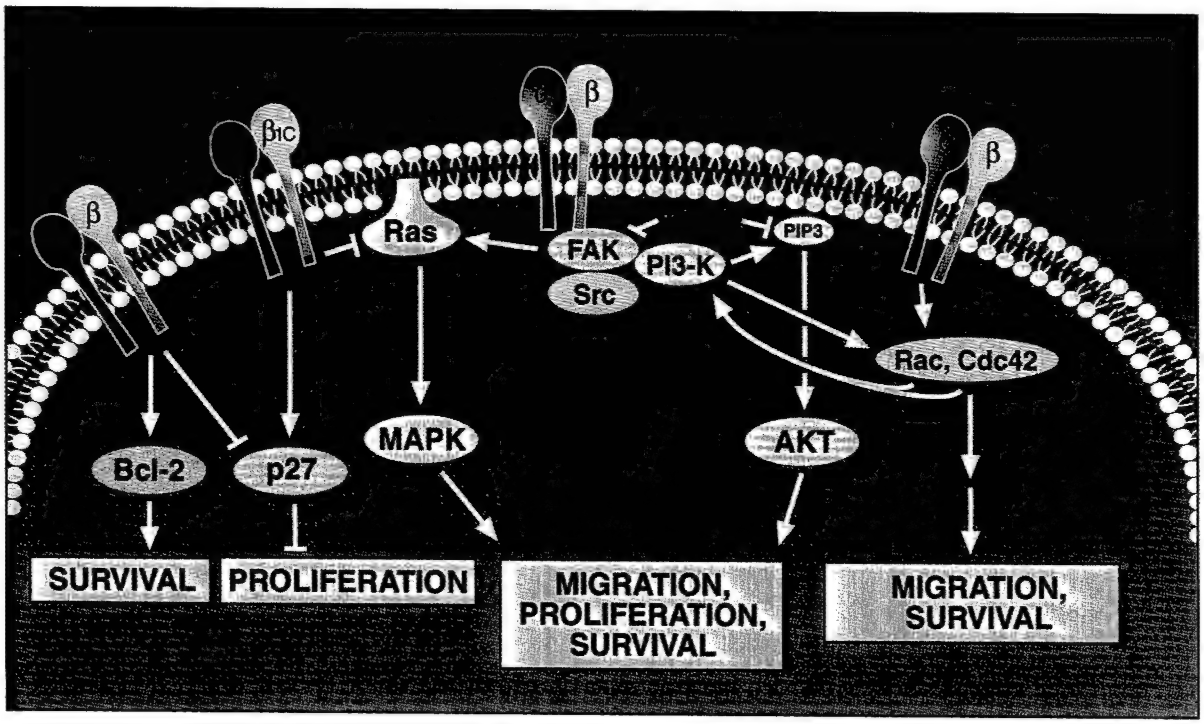

Figure 1. Signaling pathways activated by integrins and altered in prostate cancer. Schematic drawing showing the signal transduction pathways activated by integrins that control cell migration, survival and proliferation; these pathways are altered in prostate cancer. PIP3, phosphatidylinositol (3,4,5) triphosphate.

Table 3. Integrin signaling pathways that control cell migration and are altered in prostate cancer

Signaling molecule	Regulation by integrins	Alteration
FAK	Kinase activity	Up-regulation
AKT	Kinase activity	Increased kinase activity/phosphorylation
PTEN	Nd	Deletions/point mutations/ down-regulation
Ras	GTPase activity	Low frequency point mutations
MAP kinase	Kinase activity	Up-regulation and increased kinase activity/ phosphorylation

Nd, not determined.
Up-regulation, refers to protein levels.
Down-regulation, refers to protein levels.

stimuli [50]. Several studies have suggested a role for FAK in controling cell migration in response to integrin engagement or to growth factors [50]. Direct evidence on the role of FAK *in vivo* in regulating cell migration has been obtained with the generation of FAK null mice. Ablation of the FAK gene results in embryonic lethality at day 8.0–8.5 due to severe mesodermal defects [51]. Cells derived from these embryos show a decreased migration *in vitro* as compared to cells derived from wild type embryos [51,52]. In addition, FAK overexpression in chinese hamster fibroblasts and perturbation of endogenous FAK signaling in different cell types using dominant negative forms of the molecule have confirmed its involvement in controling cell motility [35,50,53,54].

In normal prostate, FAK expression is either low or absent but it is significantly increased in high-grade adenocarcinomas and in invasive and metastatic prostate cancers compared to benign prostate and low grade adenocarcinoma [55,56]. FAK association with Src, a cytoplasmic tyrosine kinase, is crucial for regulating cell migration *in vitro* [57]. Fibroblasts derived from Src, Fyn, and Yes triple knock-out mice show impaired haptotactic migration in response to fibronectin and re-expression of Src increases their ability to migrate in response to fibronectin as compared to triple knock-out fibroblasts [58]. Recently, Slack et al. [59] reported that inhibition of the FAK/Src signaling pathway significantly blocks migration of prostate carcinoma cells *in vitro*, demonstrating the crucial role exerted by these molecules in the regulation of prostate cell motility. However, analysis of Src gene alterations, protein expression and activity in prostate cancer tissues has not been performed.

5.2. PI 3-kinase/AKT

In addition to stimulating FAK, integrins can also activate the PI 3-kinase pathway [60]. PI 3-kinases comprise a family of lipid kinases activated by a wide variety of extracellular stimuli. The lipid products of PI 3-kinases, specifically phosphatidylinositol(3,4)biphosphate [PI(3,4)P_2]and (3,4,5)triphosphate [PI(3,4,5)P_3], affect cell proliferation, survival, differentiation, and migration by targeting specific signaling molecules such as the serine/threonine protein kinase B, also known as AKT [61–63]. Integrin-mediated adhesion to the ECM stimulates the production of PI(3,4)P_2 and PI(3,4,5)P_3 [64,65], the association of the p85 PI 3-kinase subunit with FAK [66] and AKT activation [64,65]. AKT plays an important role in transducing survival signals in response to several growth factors and to integrin engagement [64,67]. Recent studies have shown a significant increase in AKT kinase activity and phosphorylation associated with prostate cancer progression; specifically, the highest levels of phosphorylated AKT correlated with high Gleason grade, tumor stage III/IV and invasive cancer [68,69]. Several studies have reported that integrins control cancer cell motility through the PI 3-kinase pathway [70] which has been shown *in vitro* to be crucial for human prostate cancer cell migration [4]. Analysis of PI 3-kinase expression and activity on prostate cancer specimens is therefore needed to determine the clinicopathological significance of the PI 3-kinase pathway in prostate cancer initiation and/or progression.

5.3. PTEN

The tumor suppressor gene PTEN (or MMAC-1) encodes a dual specificity phosphatase but it has also the ability to dephosphorylate inositol phospholipids such as PI(3,4,5)P_3 and as a consequence to negatively regulate the PI 3-kinase/AKT pathway [71,72]. Interestingly, in the study mentioned above absence of PTEN expression was observed in 60% of the analyzed prostate tumors and correlated with high levels of AKT phosphorylation [68]. The PTEN gene is frequently deleted or mutated in a wide variety of human cancers and has been shown to be involved in regulation of cell migration on integrin substrates [72]. Tamura et al. [73] have shown that FAK is one of the PTEN substrates. PTEN inhibits cell migration and invasion by dephosphorylating FAK and the adapter protein Shc, thereby antagonizing integrin-triggered signaling

[72]. The PTEN gene was located at 10q23.3 [74,75]. Loss of heterozygosity (LOH) in the region 10q23.3 is present in 29–42% of clinically localized prostate cancers [76,77]. Similarly, LOH at 10q23 is present in more than 50% of metastatic prostate tumors [76–78]. PTEN is also frequently mutated or deleted in prostate cancer and prostate cancer cell lines [74,76,78,79]. PTEN is expressed at the protein level in secretory epithelia in normal adult prostate and loss of PTEN protein expression in primary prostate cancers correlates with high Gleason grade and advanced pathological stage [80]. It is, thus, conceivable that reduced PTEN expression levels might result in increased prostate cancer cell migration in vivo.

5.4. Ras/MAP kinase

Ras proteins belong to a large family of GTPases which function as signal transducers by cycling from an active GTP-bound form to an inactive GDP-bound form and activated Ras stimulates numerous signaling cascades such as the ERK1 and 2/MAP kinase pathway [81]. The Ras/MAP kinase pathway plays a pivotal role in modulating gene expression, cell cycle progression, survival and motility [82,83]. Integrin clustering has been shown to stimulate Ras GTP-loading [65,84–87] and to activate specific effectors of the Ras/MAP kinase signaling cascade [88,89] which results in increased cell proliferation, cell cycle progression and survival [90]. Some integrins exert a negative effect on the Ras/MAP kinase pathway which leads to cell cycle arrest and differentiation [91] and inhibition of cell proliferation [15]. There is evidence that cell motility is controled by integrins via a signaling cascade involving Shc and MEK1 and the MAP kinases ERK1 and 2, respectively [92,93]. Sustained activation of the Ras/MAP kinase pathway can also prevent apoptosis triggered by loss of cell–ECM contacts [94–96]. Recently it has been reported that migration and survival mechanisms promoted by integrin engagement are coordinately regulated through activation of pathways that involve ERK activity [97]. The activity and expression levels of MAP kinase are significantly higher in primary prostatic adenocarcinoma and in metastatic lesions than the levels detected in benign prostate [98–100]. Increased MAP kinase activation correlates with high Gleason score and tumor stage [98]. Since Ras mutations are uncommon in prostate cancer [46], chronic stimulation of the Ras/MAP kinase pathway is most likely achieved by alterations in the levels of upstream regulators such as integrins, growth factors and growth factor receptors during prostate cancer initiation and/or progression. Several studies suggest that integrin engagement activates members of the Rho-family of small GTPases [20,70]. Specifically, Rho, Rac, and Cdc42 have been shown to be required for cell motility [20,70]. However, analysis of Rho-family of small GTPases' gene alterations, protein expression or activity in prostate cancer tissues has not been performed.

5.5. Bcl-2

The Bcl-2 protein is a proto-oncogene that promotes cell survival [101] and is a member of a large family that consists of pro-apoptotic and pro-survival factors [102]. The Bcl-2 gene is activated by chromosomal translocation in the majority of non-Hodgkin's lymphomas and is also up-regulated in many solid tumors, indicating that it might contribute to resistance to apoptosis in response to chemotherapeutic agents and radiation therapy [102]. Adhesion to fibronectin through $\alpha_5\beta_1$ and $\alpha_v\beta_1$ and to vitronectin through $\alpha_v\beta_3$ integrins was shown to up-regulate Bcl-2 transcription and protein levels and resulted in protection from apoptosis induced by serum deprivation [103,104].

Bcl-2 protein levels are low or absent in normal prostate and Bcl-2 expression is restricted to basal cells [105]. In prostate carcinoma Bcl-2 is up-regulated and its expression correlates with hormone-refractory disease [46,105] and with poor survival [46]. Analysis of metastatic lesions obtained from prostate cancer patients after hormone treatment (hormone-refractory tumors) stained positive for Bcl-2 [106]. There is evidence for a role for Bcl-2 in promoting cancer cell motility and invasion. Overexpression of Bcl-2 increases migration and metastatic potential of breast cancer cells [107] and therefore its involvement in prostate cell migration deserves to be investigated.

6. Conclusions

This review highlights the current knowledge of the alterations that occur in prostate cancer and in prostate cancer metastases and that involve integrins and integrin-activated pathways. Although the specific functions of integrins, their ligands and their modulators in prostate cancer are not completely understood, recent publications outlining their expression pave the way for future investigations describing the role of integrin isoforms and of integrin signaling in prostate cancer cell invasion, metastatic establishment and growth.

Future research will focus on functional correlates, combining general knowledge of integrins and integrin signaling with an increasing appreciation for the role of the ECM in prostate cancer progression.

Since a single 'metastasis gene' has not been found, it is expected that multiple genetic alterations have to occur at the same time to make a cell 'metastatic'. Therefore, in addition to the disregulated expression of either integrins or integrin-activated pathways in prostate cancer, alterations of other molecules that control cell adhesion or increase either proteolysis of ECM or migration may have an important role in disease progression and metastatic spread. Among others, KAI-1, a 'prostate cancer metastasis suppressor gene' described elsewhere in details in this issue, deserves further consideration for its ability to inhibit cell migration and potentially affect integrin–ligand binding. Similarly, changes in integrin affinity, avidity, or activation state are likely to control cell–ECM interaction; additional investigations on these topics will help understanding the role of integrins in prostate cancer metastases.

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9

Expression of Heterologous Integrin Genes

Craig A. Steger, Mara Fornaro, Amy S. Woodard, Duo-Qi Zheng, and Lucia R. Languino

1. Introduction

Cell-extracellular matrix interactions are mediated predominantly by integrins, cell-surface receptors that exist as heterodimers of noncovalently associated α and β subunits *(1)*. Integrins have been implicated in organ and tissue development, normal and aberrant cellular growth, and modulation of intracellular signal-transduction mechanisms *(2–7)*. The expression of individual integrin subunits in mammalian systems has been instrumental in studies aimed at examining the structure and function of integrins and in analyzing the ability of integrins to modulate cellular functions and intracellular signaling (for review, *see* **ref. *8***).

High levels of expression of integrin heterodimers have been obtained in several cell types under the control of either CMV or SRα or SV40 or the mouse metallothioneine-inducible promoter. More recently, inducible systems based on components of the tetracycline-resistance operon or on nonmammalian steroid hormones have also been generated to modulate integrin gene activity *(9,10)*; these systems have the remarkable advantage of a tighter control of expression versus previously used inducible systems.

Several strategies have emerged to introduce DNA into eukaryotic cells; however, an ideal procedure valid in all cases is not available. This chapter will describe two approaches successfully used in our laboratory to transfect integrin subunit cDNAs into mammalian cells: electroporation, that utilizes short bursts of high-voltage electricity *(11)*, and lipofection, that utilizes lipid-DNA complexes. It should be noted that the efficacy of each method may vary in different applications, thus their use should be tested for the investigator's

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specific needs; low-expression levels of the desired integrin could be a reflection of protein instability or difficulties with transfection. The latter should be tested by a readily assayable indicator gene, such as β-galactosidase or luciferase integrin. It should also be noted that because integrins are heterodimeric complexes, an exogenously expressed subunit must pair with an endogenous subunit to be correctly processed and transported to the cell surface. If endogenous subunits are not available to heterodimerize with the transfected subunit, cotransfection of both α and β subunit cDNAs should be performed.

Although we will selectively discuss protocols for transient transfection of Chinese hamster ovary (CHO) cells, the same procedures can be used to generate either stable or transient transfectants of various cell types. An immunological and a functional approach for the detection of the successfully expressed integrin heterodimer will also be described in this chapter.

2. Materials

2.1. Transient Transfection of CHO Cells

2.1.1. Electroporation

1. Subconfluent (70%) CHO cells, approx 1×10^7 cells/electroporation.
2. CHO growth medium: 500 mL DMEM supplemented with high glucose (Life Technologies, Gaithersburg, MD), 5 mL of 200 m*M* L-glutamine (Gemini BioProducts, Calabasas, CA), 5 mL penicillin-G (10,000 U/mL) Streptomycin (10,000 mg/mL) (Gemini BioProducts), 5 mL of 200 m*M* nonessential amino acids (Life Technologies) and 10% heat-inactivated fetal bovine serum (Gemini BioProducts). Store growth medium at 4°C.
3. Phosphate-buffered saline (PBS) *(12)*. Sterilize and store at 4°C.
4. Trypsin-EDTA (0.05% trypsin and 0.53 m*M* EDTA) (Life Technologies). Store at 4°C.
5. 0.4% Trypan blue stain (Life Technologies) diluted 1:2 in PBS.
6. Hemocytometer (Reichert-Jung, Horsham, PA).
7. Electroporation buffer: 10 m*M* sodium phosphate and 150 m*M* sodium chloride at pH 7.4. Sterilize and store at 4°C.
8. Salmon sperm DNA (Sigma, St. Louis, MO) that has been sonicated and resuspended at 10 mg/mL *(12)*. Store at –20°C.
9. Integrin cDNA subcloned in a mammalian expression vector and purified with either commercially available Maxi-prep kits or by cesium-chloride purification *(12)*. Cesium-chloride-purified DNA must be dialyzed against sterile-deionized water. Visualize DNA with ethidium bromide. Store at –20°C (*see* **Note 1**).
10. 150-mm tissue-culture plates (Falcon, Franklin Lakes, NJ).
11. 15-mL conical tubes (Corning, Corning, NY).
12. Electroporation cuvets, 0.4-cm electrode gap (Bio-Rad, Hercules, CA).
13. Gene Pulser II, electroporator with capacitance extender (Bio-Rad).

2.1.2. Lipofection

1. Mammalian expression vector DNA of interest, purified as described in **Subheading 2.1.1., step 9**.
2. Approximately 40–70% subconfluent CHO cells in 60-mm tissue culture plates.
3. CHO growth medium (refer to **Subheading 2.1.1., step 2**).
4. Lipofectin (Life Technologies).
5. OptiMEM (Life Technologies).
6. Sterile 1.5-mL microcentrifuge tubes (USA/Scientific, Ocala, FL).
7. 60-mm tissue culture plates (Corning).

2.2. Detection of Exogenous Integrins at the Cell Surface

2.2.1. Cell Staining for Flow Cytofluorometric Analysis

1. CHO growth medium (refer to **Subheading 2.1.1., step 2**).
2. PBS (refer to **Subheading 2.1.1., step 3**).
3. Trypsin-EDTA (refer to **Subheading 2.1.1., step 4**).
4. Trypan blue (refer to **Subheading 2.1.1., step 5**).
5. Hemocytometer.
6. 5-mL round-bottom tubes (Falcon).
7. Species-specific antibody (Ab) that recognizes the extracellular domain of the exogenously expressed integrin (primary Ab, 1° Ab) and that does not cross-react with the hamster protein.
8. Negative control Ab. Isotype matched Abs either against a cytoplasmic protein, or against a surface protein that is not expressed by CHO cells or nonimmune IgG can be used.
9. Secondary Ab (2° Ab) conjugated to a fluorophore (e.g., fluorescein isothiocyanate, FITC).
10. 3% Paraformaldehyde (Sigma) stock solution in PBS. Working solution is diluted 1:50 (*see* **Note 2**).

2.2.2. Surface Iodination and Immunoprecipitation

1. Approximately 2×10^7/mL CHO cells.
2. Sterile PBS (refer to **Subheading 2.1.1., step 3**).
3. Sterile 100 m*M* $CaCl_2$ (J.T. Baker, Phillipsburg, NJ).
4. Sterile 100 m*M* $MgCl_2$ (J.T. Baker).
5. Lactoperoxidase (Sigma) at 3 mg/mL in PBS. Store in aliquots at –20°C.
6. 30% stock solution of hydrogen peroxide (H_2O_2). The working solution is prepared fresh and diluted to 0.24% in PBS. Store on ice (*see* **Note 3**).
7. 1 mCi sodium iodine-125 ($Na^{125}I$). Store behind lead shielding at room temperature (*see* **Note 4**).
8. CHO growth medium (refer to **Subheading 2.2.1., step 2**).
9. Trypan blue (refer to **Subheading 2.2.1., step 5**).
10. Hemocytometer.
11. 15-mL conical tube (Corning).

12. Lysis buffer: 20 m*M* Tris-HCl at pH 8.0 (American Bioanalytical, Natick, MA), 1% Triton-X 100 (Sigma), 10% glycerol (J.T. Baker), 150 m*M* NaCl (J.T. Baker), 1 m*M* PMSF (Life Technologies), 10 µg/mL aprotinin (Sigma), and 10 µg/mL leupeptin (Calbiochem, La Jolla, CA).
13. 1.5-mL microcentrifuge tubes.
14. Nonimmune Ab.
15. Ab specific for the exogenously expressed integrin.
16. Protein A sepharose (Sigma).
17. Rotating platform.
18. SDS sample buffer: 50 m*M* Tris-HCl at pH 6.8 (American Bioanalytical), 2% SDS (American Bioanalytical), 10% glycerol, and 0.1 *M* dithiothreitol (Bio-Rad) and 0.1% bromophenol blue (Bio-Rad).
19. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) apparatus.
20. Coomassie stain solution: 40% methanol, 10% acetic acid, and 0.02% Coomassie blue.
21. Gel dryer.
22. Autoradiography cassette with intensifying screens and film.

2.3. Assaying for Functional Integrin Expression

2.3.1. Cell Adhesion Assay

1. CHO Growth medium (refer to **Subheading 2.1.1., step 2**).
2. PBS (refer to **Subheading 2.1.1., step 3**).
3. Trypsin-EDTA (refer to **Subheading 2.1.1., step 4**).
4. Trypan blue (refer to **Subheading 2.1.1., step 5**).
5. Hemocytometer.
6. Linbro®/Titertek® microtitration 96-well plate (ICN Biomedicals, Aurora, OH).
7. Assay Buffer: DMEM (refer to **Subheading 2.1.1., step 2**) supplemented with 1% bovine serum albumin (BSA) (Sigma).
8. 3% Paraformaldehyde (refer to **Subheading 2.1.1., step 10**).
9. 0.5% Crystal violet (Sigma) dissolved in deionized water and filtered through filter #1 paper (Whatman, Fairfield, NJ) prior to using.
10. 96-well plate reader with 630-nm wavelength filter.

3. Methods

3.1. Transient Transfection of CHO Cells

The following methods: electroporation *(13)* and lipofection have been used to transiently transfect CHO cells with an integrin cDNA, subcloned into a mammalian expression vector. In our laboratory, the best results have been achieved using plasmids that have been purified by cesium-chloride gradients. However, commercially available maxi-prep kits (e.g., Promega, Madison, WI or Qiagen, Chatsworth, CA) can also be used to purify the plasmid.

3.1.1. Electroporation

1. Grow CHO cells to subconfluent levels (50–70%) and subculture the cells 24 h before electroporation. Approximately 1×10^7 cells will be needed for each electroporation.
2. The following day, wash cells once with sterile PBS.
3. Detach cells by incubating with trypsin-EDTA for 3–5 min.
4. Neutralize trypsin-EDTA with an equal amount of CHO growth medium and transfer the cells into a 15-mL conical tube.
5. Remove 10 µL of cells and dilute into 90 µL 0.2% trypan blue. Count the cells using a hemocytometer, and determine the total number of cells.
6. Centrifuge the remaining cells for 4 min at 300*g*.
7. Wash the cells once with sterile PBS and place on ice.
8. Add 2–100 µg plasmid DNA to sterile electroporation cuvet (*see* **Note 5**).
9. Add 50 µg salmon sperm DNA to each cuvet.
10. Equilibrate the volume of each cuvet using sterile water.
11. Resuspend 1×10^7 cells/cuvet in electroporation buffer, so that the final volume of each cuvet does not exceed 500 µL (*see* **Note 6**).
12. Add cells to the cuvet and incubate on ice for 15 min.
13. Dry the outside of the cuvet thoroughly (*see* **Note 7**).
14. Set the electoporator to 350 V and 950 µF.
15. Electroporate the cells.
16. Allow the cells to recover on ice for 15 min.
17. Plate the cells into a 150-mm tissue-culture dish with CHO growth medium and incubate at 37°C with 5% CO_2 in a humidified incubator (**Notes 8** and **9**).
18. Replace the medium 24 h later and incubate the cells with 5% CO_2 in a humidified incubator until the cells are to be analyzed for integrin expression. (*See* **Notes 10** and **11**.)

3.1.2. Lipofection

1. Place 0.5–2 µg purified plasmid DNA into a 1.5-mL microcentrifuge tube and bring the final volume to 100 µL with OptiMEM and gently mix by pipetting up and down several times (*see* **Note 5**).
2. Place 0.5–10 µg Lipofectin in a 1.5-mL microcentrifuge tube and bring the volume to 100 µL with OptiMEM and gently mix by pipetting up and down several times (*see* **Note 12**).
3. Incubate both 1.5-mL microcentrifuge tubes for 30 min at room temperature.
4. Combine the contents of both tubes (gently mixing) and incubate for 10 min at room temperature.
5. Wash the CHO cells 2× with OptiMEM (*see* **Notes 13** and **14**).
6. Add 800 µL OptiMEM to the 1.5-mL microcentrifuge tube that contains both the DNA and lipofectin and gently mix.
7. Add the solution from **step 6** drop wise to the CHO cells.
8. Incubate the cells for 7–14 h at 37°C with 5% CO_2 in a humidified incubator (*see* **Note 15**).

9. Replace transfection solution with CHO growth medium and incubate as in **Subheading 3.1.1., step 18.**

3.2. Detection of Exogenous Integrin at the Cell Surface

Integrin expression can be analysed using Ab5 specific to the heterologous integrin by flow cytofluorometric analysis (**Subheading 3.2.1**) or by immunoprecipitation of surface-iodinated transfected cells (**Subheading 3.2.2**). Adhesion assays should also be performed to confirm that the expressed integrin is functionally active (**Subheading 3.3.1**). In our transient transfection system, maximal exogenous integrin expression is observed at 48 h after the transfection, however, it is suggested to determine the optimal time for each cell type and integrin of interest.

3.2.1. Flow Cytofluorometric Analysis

1. Remove medium from the CHO cells and wash once with sterile PBS.
2. Detach the cells from the tissue-culture plate by incubating the cells with trypsin-EDTA for 3–5 min.
3. Neutralize the trypsin with an equal volume of CHO growth medium.
4. Remove a 10-µL aliquot of cell suspension and add it to 90 µL of 0.2% trypan blue.
5. Count the cells using a hemocytometer and determine the total number of cells.
6. Centrifuge the cells at 300*g* for 4 min.
7. Resuspend the cells in CHO growth medium, and then place 5×10^5 cells, each, into two separate 5-mL round-bottom tubes (*see* **Note 16**).
8. Repeat **step 6**.
9. Aspirate the supernatant.
10. Resuspend the cells in 50 µL of CHO growth medium containing the 1° Ab (*see* **Note 17**).
11. Incubate the cells for 30 min on ice.
12. Add 1 mL of CHO growth medium to each tube, and repeat **step 6**.
13. Wash the cells 2× with 1 mL CHO growth medium.
14. Resuspend the cells in 50 µL of medium containing the 2° Ab (*see* **Notes 18** and **19**).
15. Repeat **steps 11** and **12**.
16. Wash the cells once with CHO growth medium and once with sterile PBS.
17. Resuspend the cells in 0.06% paraformaldehyde in PBS and perform flow cytofluorometric analysis.

3.2.2. Surface Iodination and Immunoprecipitation

1. Remove medium from the CHO cells and wash once with sterile PBS.
2. Detach the cells from the tissue-culture plate by incubating the cells with trypsin-EDTA for 3–5 min.
3. Neutralize the trypsin with an equal volume of CHO growth medium.
4. Remove a 10 µL aliquot of cell suspension and add it to 90 µL 0.2% trypan blue.
5. Count the cells using a hemocytometer and determine the total number of cells.
6. Centrifuge the cells at 300*g* for 4 min.

7. Wash the cells 3X with PBS.
8. Resuspend the cells in PBS, containing 1 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$, at a final concentration of 2×10^7 cells/mL in a 15-mL conical tube.
9. Add 200 µL of lactoperoxidase to the cells.
10. Add 1 mCi $Na^{125}I$ to the cells (*see* **Notes 4, 20,** and **21**).
11. Add 40 µL 0.24% H_2O_2 to the cells.
12. Incubate cells on ice for 5 min, with gentle vortexing after each minute.
13. Add 40 µL of 0.24% H_2O_2, and repeat **step 12**.
14. Add 10 mL PBS containing 0.5 mg/mL tyrosine to bind all of the remaining free $Na^{125}I$ (*see* **Note 22**).
15. Incubate the cells for 5 min at room temperature (*see* **Note 23**).
16. Wash cells 3× with 10 mL of PBS.
17. Determine the volume of the cell pellet, and lyse with an equal volume of lysis buffer at 4°C for 30 min.
18. Centrifuge at 14,000*g* for 30 min, and transfer the supernatant to a fresh tube.
19. Count an aliquot of the lysate in a gamma counter to determine the amount of radioactive material in the starting lysate. Use counts per minute, cpm.
20. Incubate lysate with nonimmune serum followed by Protein A-sepharose for 30 min each, at 4°C (*see* **Note 24**).
21. Centrifuge the Protein A-sepharose beads at 5000*g* for 5 min.
22. Repeat **steps 20** and **21**.
23. Repeat step **19**.
24. Remove an equal amount of cpms, for each immunoprecipitation, from the supernatant and incubate it with an Ab that is specific for the transfected integrin (*see* **Notes 25** and **26**). Using equal cpms is an important control when comparing two different immunoprecipitations to each other.
25. Incubate this reaction overnight at 4°C (*see* **Note 24**).
26. Add an equal volume of protein A Sepharose as in **step 20**, and incubate for 2–4 h at 4°C (*see* **Note 24**).
27. Wash the immunocomplexes 5X with lysis buffer (*see* **Note 27**).
28. Resuspend the immunocomplexes in SDS sample buffer.
29. Heat samples to 95°C for 3–5 min.
30. Separate proteins on a 7.5% SDS-PAGE.
31. Coomassie stain and dry the SDS-PAGE gel to fix and visualize the molecular weight markers if they were not prestained or radiolabeled.
32. Visualize the labeled proteins by autoradiography (*see* **Note 28**).

3.3. Assaying for Functional Integrin Expression

3.3.1. Cell Adhesion

1. Coat Linbro® 96-well plates as described *(14)*, with 100 µL per well of the testing substrate in PBS at 4°C overnight, or at 37°C for 1 h (*see* **Note 29**).
2. Discard the substrate and rinse the plates 3× with 200 µL PBS per well.
3. Block plates with 100 µL of blocking buffer per well, and incubate at 37°C for 1 h.
4. Remove medium from the CHO cells and wash once with sterile PBS.

5. Detach the cells from the tissue culture plate by incubating the cells with trypsin-EDTA for 3–5 min.
6. Neutralize the trypsin with an equal volume of CHO growth medium, and transfer the cells to a 15-mL conical tube.
7. Remove a 10-µL aliquot of cell suspension and dilute into 90 µL 0.2% trypan blue.
8. Count the cells using a hemocytometer and determine the total number of cells.
9. Centrifuge the cells for 4 minutes at 300*g*.
10. Wash the cells once with sterile PBS.
11. Wash the cells 3× with assay buffer and resuspend the cells in the appropriate volume of assay buffer.
12. Rinse the coated and blocked plates 3× with 200 µL of PBS per well.
13. Place a 100-µL aliquot of cell suspension into the wells and incubate at 37°C and 5% CO2 in a humidified incubator (*see* **Note 30**).
14. Gently wash the wells 2× with 200 µL of PBS.
15. Fix the adherent cells with 100 µL of 3% paraformaldehyde at 4°C for 30 min (*see* **Note 2**).
16. Gently wash the wells 2× with 200 µL of PBS.
17. Stain the cells with 100 µL of 0.5% crystal violet, and incubate at room temperature for 2 h or overnight.
18. Aspirate the crystal violet, wash the wells 2× with 200 µL of PBS, and aspirate any remaining liquid from the wells.
19. Read OD_{630nm}.

4. Notes

1. Use appropriate safety precautions when using ethidium bromide.
2. When working with paraformaldehyde, gloves should be used at all times.
3. 30% Hydrogen peroxide can cause burns, so proper precautions should be taken when working with concentrated peroxides.
4. ^{125}I is a volatile compound that will readily incorporate into the thyroid. Consult your institutions and Nuclear Regulatory Commission guidelines for working with this material.
5. Concentrations of purified integrin cDNA necessary for successful transfection will have to be determined in each case.
6. Each electroporation must contain 1×10^7 cells, and the final volume of each cuvette should not exceed 500 µL, therefore, the volume of DNA and water must be taken into consideration prior to resuspending the cells.
7. To avoid electrical shocks, the outside of the cuvet must be dried thoroughly.
8. Cellular debris after the electroporation will be observed.
9. It is essential to dilute the cells into at least 6 mL of growth medium.
10. The medium needs to be changed 24 h after the electroporation to remove the cellular debris and dead cells.
11. Surface expression of the transfected integrin is easily detectable at 48 h after electroporation, however, the optimal time of expression for each integrin may vary for each cell type and integrin.

12. The volume of lipofectin needed must be determined for the cell type and integrin of interest.
13. Cells remain attached to the tissue-culture plate.
14. Cells should be subconfluent in a 60-mm dish (approx 40–70%), however the density should be adjusted in a cell-type-dependent manner.
15. Incubation time is dependent upon cell type.
16. Two separate tubes of cells will be needed for the staining. In one tube, the cells will be stained with an isotype-matched control 1° Ab (**Subheading 2.2.1., step 8**), and the other tube will be stained with a 1° Ab that specifically recognizes the transfected integrin (Subheading **2.2.1., step 7**).
17. The specific 1° Ab must recognize the extracellular domain of the exogenously expressed integrin. Either monoclonal or polyclonal Abs can be used and the concentration of the Ab will vary accordingly. It is important to use a 1° Ab that will not cross-react with endogenous integrins. It is also important to determine the saturating concentration of both the 1° and 2° Ab to be used.
18. Select a 2° Ab for its species specificity against the 1° Ab. This Ab must be conjugated to a fluorophore such as FITC. The use of a 1° Ab that is directly conjugated to a fluorophore is possible.
19. Restrict the samples' exposure to light because of the sensitivity of fluorophores.
20. You must work in a properly authorized fume hood and behind protective lead shielding when working with an opened vial containing free $Na^{125}I$.
21. All waste, both liquid and solid, must be disposed of as radioactive waste throughout the remainder of the experiment.
22. Iodination reaction can also be stopped with 10 mL of PBS containing 0.02% sodium azide to inhibit lactoperoxidase activity, but this will not bind the free $Na^{125}I$.
23. After quenching the free $Na^{125}I$, it is safe to work outside the hood, but always following the proper precautions when working with radioactive material.
24. All incubations during the immunoprecipitation should be done with mild agitation on a rotating platform. Samples should be shielded with lead foil.
25. If using a mouse Ab it may be necessary to use either an intermediate incubation with rabbit anti-mouse IgG.
26. A species-specific Ab for the transfected integrin should be used. Avoid the use of Abs that will cross-react with endogenous integrins.
27. For immunoprecipitations using polyclonal Abs, the first three washes should be performed with lysis buffer, supplemented with 350 m*M* NaCl, followed by two washes using lysis buffer containing 150 m*M* NaCl. Immunoprecipitations using MAbs should be washed 5X with the lysis buffer as described in the material section.
28. Dried gel should be exposed to film in the presence of intensifying screens at –70°C. The length of the exposure must be determined for each experiment.
29. Duplicate wells for the adhesion assay should be coated with an extracellular matrix molecule (ECM) that specifically binds for the transfected integrin or an Ab that will recognize the exogenous integrin. The concentration of ECM used for coating will have to be determined for each cell type.

30. Cell number per well will vary according to cell type, as will the length of time needed for cell attachment.

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Monday
October 14

PLENARY
SESSION #3

10:20 – 10:55 am

Role of β1 integrins and β1 – downstream effectors in prostate cancer cell adhesion and proliferation

Lucia R. Languino, Cancer Biology, University of Massachusetts Medical School, Worcester, MA

The interactions between cancer cells and extracellular matrix proteins are mediated by integrins that have emerged as critical modulators of cell adhesion, proliferation, migration and intracellular signaling. The pathological consequences of integrin-deregulated expression in prostate cancer are the focus of our current investigations. Our findings show that the β1 integrins are differentially expressed in normal and neoplastic cells and that their expression in genetically engineered prostate cancer cells significantly affects prostate tumor growth *in vivo*. *In vitro*, we demonstrate that the β1 integrins modulate cell adhesion and proliferation via specific signaling events and via regulation of gene expression. Studies in our laboratory aimed to identify downstream effectors of β1 have unraveled a novel IGF (insulin-like growth factor)II - mediated pathway regulated by β1 integrins; this pathway involves regulation of gene expression and results in significant changes in cell adhesion. Thus, given their deregulated expression in neoplastic cells and their ability to control multiple downstream signals, the β1 integrins and their downstream effectors provide exciting opportunities for new approaches to prostate cancer therapy.

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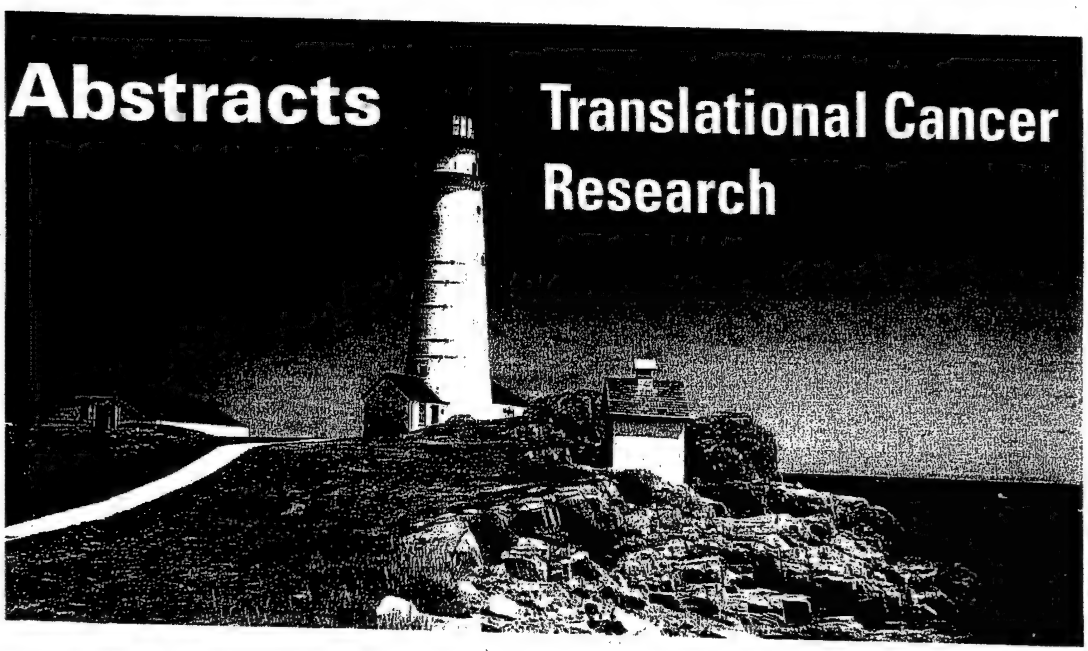

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ADHESION MOLECULES

Lucia R. Languino, Loredana Moro, Tom Manes, Mara Fornaro and Duo-Qi Zheng
Yale University, Department of Pathology, New Haven, CT

The interactions between cancer cells and extracellular matrix proteins are mediated by integrins, that have emerged as critical modulators of cell adhesion, proliferation, migration and intracellular signaling. The pathological consequences of integrin deregulated expression in cancer are the focus of current investigations. The β1 and β3 integrins are differentially expressed in normal and neoplastic cells. Our recent findings show that their expression plays a pivotal role in modulating prostate cancer cell functions *in vivo*, since they significantly affect prostate tumor growth. Using genetically engineered and primary prostate cancer cells, we have found that, *in vitro*, the β1 and β3 integrins modulate cell adhesion and motility via activation of specific signaling events and via regulation of gene expression. Studies in our laboratory aimed to identify downstream effectors of β1 and β3 integrins have unraveled two novel pathways regulated by β1 and β3 integrins that involve changes in gene expression; respectively, an IGF (insulin-like growth factor)II - mediated pathway that controls cell adhesion and a cdc2 (cyclin-dependent kinase 1, cdk1) - mediated pathway that controls cell migration. Therefore, given their deregulated expression in neoplastic cells and their ability to control multiple downstream signals, the β1 and β3 integrins and their downstream effectors provide exciting opportunities for new approaches to cancer therapy.

"Predictive Oncology and Intervention strategies"Conference

Feb 9-12 (2002), Paris, France

Regulation of Cancer Cell Proliferation and Survival by β1 Integrins.

Mara Fornaro, Duo-Qi Zheng,, Michela Manzotti #, Giovanni Tallini, and Lucia R. Languino
Department of Pathology, Yale University School of Medicine, 310 Cedar Street, New Haven, CT 06520 and # Department of Pathology, European Institute of Oncology, Via Ripamonti 435, 20141 Milan, Italy.

AIM: Alterations of integrin expression in cancer affect tumor growth. The β1C integrin is a cytoplasmic domain variant of the β1 subfamily. At variance with β1A, β1C inhibits *in vitro* cancer cell proliferation and *in vivo*, it is selectively downregulated in prostatic adenocarcinoma. A potential correlation of expression of β1C and of the cyclin kinase inhibitor $p27^{kip1}$ *in vivo* in benign and neoplastic prostate tissues as well as β1C downstream signaling pathways that control cancer cell proliferation and survival were studied. **METHODS:** Tissue microarray technology, conventional immunohistochemistry and immunoblotting analysis were employed to investigate the expression of β1C and $p27^{kip1}$ in prostatic adenocarcinoma. Biochemical assays were performed to analyze β1C modulation of signaling pathways involved in the control of cell cycle progression and survival. **RESULTS:** A very high correlation of β1C and $p27^{kip1}$ expression was found in 93% of benign cells and in 84-91% of neoplastic cells of the analyzed specimens ($p<0.0001$). In 75% of the specimens analyzed, both β1C and $p27^{kip1}$ were downregulated in tumor areas in comparison to benign counterparts. In contrast to β1A, forced expression of β1C *in vitro* was accompanied by an increase in $p27^{kip1}$ levels, by inhibition of cyclin A-dependent kinase activity and of the Ras/MAP kinase pathway.

Furthermore, $\beta 1C$ sensitized cells to drug-induced apoptosis. $\beta 1C$ inhibitory effect on cell proliferation and survival was completely prevented by $p27^{kip1}$ antisense or by expression of activated Ras and MAP kinase. **CONCLUSIONS:** These results indicate that $\beta 1C$ may be a sensitive prognostic indicator of potential high clinical value to predict therapy and patient survival for prostatic adenocarcinoma and that the $\beta 1C$ integrin, via a unique signaling mechanism, controls cancer cell proliferation and survival. **KEY WORDS:** adhesion, cancer, integrin cytoplasmic domain, cyclin-dependent kinase inhibitors.

Abstracts of papers presented
at the 2001 meeting on

TYROSINE PHOSPHORYLATION & CELL SIGNALING

May 16–May 20, 2001

Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

A NOVEL AUTOCRINE MECHANISM ACTIVATED BY β_1 INTEGRINS THAT SUPPORTS CELL ADHESION VIA IGFII AND TYPE 1 IGF RECEPTOR

Moro, L., Fornaro, M., and L. R. Languino
From the Department of Pathology, Yale University School of Medicine, New Haven, CT 06520

Integrins and growth factor receptors are known to modulate signaling pathways either independently or synergistically. Using β_1 integrin cell transfectants, we show that β_{1C}, a β_1 cytoplasmic variant, mediates cell adhesion to laminin-1, whereas the β_{1A} cytoplasmic variant poorly supports cell adhesion to laminin-1. The increased cell adhesion to laminin-1 mediated by β_{1C} is caused by upregulation of IGF (insulin-like growth factor) II at the mRNA and protein levels, and is significantly inhibited by antibodies to IGFII and to the type 1 IGF receptor (IGF-IR). In β_{1C}–cells, IGFII increased levels cause PI3-kinase activation that, as evaluated by the use of dominant negative forms of PI3-kinase, is the only downstream signal that controls cell adhesion to laminin-1. In contrast, the β_{1A} variant does not upregulate IGFII levels and shows minimal binding to laminin-1. Finally, the IGF-IR shows the ability to associate with β_{1A}, whereas it does not associate with β_{1C} and is constitutively phosphorylated on tyrosine in β_{1C}–expressing cells. In conclusion, we show here that the β_1 integrin extracellular binding domain is unable to mediate cell adhesion to laminin unless the following cascade of events is activated: upregulation of IGFII levels, phosphorylation on tyrosine of IGF-IR and activation of PI3-kinase. Thus, we describe here a novel autocrine mechanism that supports cell adhesion via IGFII and its binding partner, IGF–IR, and that is selectively regulated by the β_1 integrin cytoplasmic domain in a PI3-kinase dependent manner.

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mediated by dynamitin□fs interaction with PKC (protein kinase C) substrate MacMARCKS (Macrophage-enriched myristoylated alanine-rich protein kinase C substrate) (JBC, 275:23948, and JBC 276:12979). Previous researches have shown that both MacMARCKS and microtubules are directly involved in releasing integrin molecules from cytoskeletal constraint and thus leading to integrin activation and cell adhesion. However, there is no direct evidence that dynamitin or dynactin complex is involved in integrin activation. Thus, we measured the lateral mobility of β_2 integrin molecules in macrophage cells, which reflects the degree of cytoskeletal constraint. Using the single particle tracking method as tool, we found that integrin molecules in cells expressing the fusion protein of CFP(cyan fluorescence protein)-dynamitin or CFP-MB (MacMARCKS binding domain peptide of dynamitin) showed a six-fold increase in diffusion coefficient (2.05□)0.69 $X10^{-10}$ and 2.07□)0.78$X10^{-10}$ respectively) than the cell expressing control CFP only (0.32□)0.09$X10^{-10}$), suggesting that disturbance in dynamitin function dramatically altered the cytoskeletal constraint on β_2 integrin molecules. This data also correlates with the effects of dynamitin mutants on the cells adhesion and its interaction with MacMARCKS protein. Therefore we conclude that the dynamitin (and maybe the whole dynactin complex as well) is part of the cytoskeletal constraint that locking β_2 integrin in the inactive form. (This study was supported by NIH grant GM54715 to Jianxun Li).

2554

The Signal Transduction Pathways Regulating the Cytoskeletal Constraint on β_2 Integrin through Ca^{2+}, PKC and Calmodulin

Ximing Zhou, Tianquan Jin, Jianxun Li, Oral Biology, University of Illinois at Chicago

Cytoskeletal constraint on β_2 integrin molecules is essential for keeping these molecules in an inactive state. Activation of protein kinase C (PKC) induces integrin activation by relaxing the cytoskeletal constraint, which is represented by an increased lateral mobility and clustering of integrin molecules. While our previous studies have demonstrated that MacMARCKS, actin filaments and microtubules are all parts of this cytoskeletal constraint on β_2 integrin (JBC, 275:20217), many other molecules may also be involved. We therefore examine whether Ca^{2+} influx and calmodulin are also involved in the regulation of this cytoskeletal constraint. Here we report that receptor-mediated Ca^{2+} influx induced by Thapsigargin, or Ca^{2+} influx induced by Ca^{2+} ionophore A23187, both resulted in an increase of the lateral mobility of β_2 integrin molecules on the cell membrane. Both inhibitors of PKC and of calmodulin inhibited Ca^{2+} effect on integrin mobility. Meanwhile, inhibitor of Ca2+ influx had no effect on phorbol esters induced integrin mobility, suggesting that Ca^{2+} signal is upstream of PKC. Furthermore, calmodulin was found downstream of PKC because calmodulin inhibitors blocked both Ca^{2+} induced and phorbol esters-induced integrin mobility. It is also observed that Ca^{2+} influx-induced integrin mobility coincide with integrin activation-dependent tyrosine phosphorylation of paxillin, suggesting that Ca^{2+} induced integrin mobility indeed corresponds to the activation of β_2 integrin. Thus, we conclude that both Ca^{2+} and calmodulin are parts of the regulation of the cytoskeletal constraint on the β_2 integrin, and the signal transduction goes from Ca^{2+} to PKC and then to calmodulin, and finally to actin filaments and microtubules. (This study was supported by NIH grant GM54715 to Jianxun Li).

2555

Integrin Clustering Independent and Clustering Dependent Phases in FAK-Mediated Signaling

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While it is clear that integrin mediated adhesion can stimulate intracellular signaling pathways, the mechanistic link between the ligand binding by integrin and the initiation of the signaling processes remains unknown. A kinetic approach was used to link a5b1 mediated adhesion to fibronectin with downstream phosphorylation events in HT1080 cells. Both the strength of adhesion, as measured using the spinning disc device, and the proportion of a5 and b1 bound to fibronectin increased linearly with fibronectin density. The linearity indicates that clustering does not contribute to adhesion strength under these conditions. There was an increase in phosphorylation of Y397 that was linear and directly proportional to the adhesion strength. This phosphorylation provides an intracellular readout of the strength of a5b1-mediated adhesion. In a comparison of HT1080, IMR90 and CEF, each line showed the same dependence of FAK Y397, but not other phosphorylation sites, on adhesion strength. In contrast, clustering of a5b1 with specific antibodies has little effect on Y397 phosphorylation but produced large increases in phosphorylation of Y861 and other src-dependent sites on FAK. In the non-clustered situation the bound src could only phosphorylate the FAK to which it was bound. Thus, ligand binding induced a direct response in the level of pY397, clustering of integrin also clusters FAK and increases the phosphorylation of sites involved in downstream signaling and provides for signal amplification.

2556

Regulation of Rac Stimulated Jun Kinase Signaling by Nischarin, an Integrin α5 Subunit Binding Protein

Peter J. Reddig[1], Rudy L. Juliano[2], [1]Pharmacology & Lineberger Comprehensive Cancer Center, U. of North Carolina-Chapel Hill, 906A Mary Ellen Jones CB# 7365, Chapel Hill, NC 27599, [2]Pharmacology, U. of North Carolina-Chapel Hill

Nischarin is an integrin α5 subunit binding protein which specifically binds to the cytoplasmic domain of integrin α5. Nischarin can regulate cell motility, cytoskeletal architecture, and signaling from the Rho GTPase, Rac. Stimulation of lamellipodia formation and c-fos promoter activity by activated Rac is suppressed by elevation of intracellular levels of Nischarin (Alahari, S., et. al., JCB 151:1141). The effect of Nischarin on another Rac downstream pathway, the Jun kinase (JNK)signaling pathway, was examined. Co-expression of a constitutively active Rac (Rac Q61L) with Nischarin in NIH-3T3 cells enhances Rac stimulation of a luciferase reporter gene dependent on JNK for stimulation. Co-expression of Nischarin with activated protein kinase A did not significantly effect the expression of a protein kinase A dependent reporter gene. Stimulation of JNK1 kinase activity, assayed by JNK1 phosphorylation of c-Jun, by activated Rac Q61L was also enhanced by co-expression of Nischarin. Thus, Nischarin can positively modulate Rac stimulation of the JNK pathway.

2557

Positive regulation of cell-cell and cell-substrate adhesion by protein kinase A

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Integrin receptor activation is an important regulatory mechanism for cell-substrate and cell-cell adhesion. We have previously described the effects of an activating anti-β_1 monoclonal antibody (mAb), 12G10, that can specifically and rapidly induce both cell-substrate and cell-cell adhesion (Exp. Cell Res. 263:65-76, 2001). In this study, we investigated signaling pathways activated by mAb 12G10 that are required to induce integrin-mediated cell-cell and cell-substrate adhesion. We have found that the cAMP-dependent protein kinase (PKA) is required for both mAb 12G10-induced cell-cell and cell-substrate adhesion of HT-1080 cells. Binding of mAb 12G10 to β_1 integrins stimulates an increase in intracellular cAMP levels and PKA activity, and a concomitant shift in the localization of the PKA type II regulatory subunits from the cytoplasm to areas where integrins expressing the 12G10 epitope are located. Two processes required for HT-1080 cell-cell adhesion, integrin clustering and F-actin polymerization, are also both dependent on PKA. Furthermore, our results suggest that the increase in intracellular cAMP levels and PKA activity, following activation of β_1 integrins with mAb 12G10, is caused by a decrease of phosphodiesterase enzyme activity. MAb 12G10-induced cell-cell adhesion was mimicked by a combination of clustering either α_2 or β_1 integrins and elevating PKA activity with Sp-cAMPS or forskolin, potent activators of PKA, or rolipram, a selective inhibitor of phosphodiesterase type IV. Taken together, our data suggest that PKA plays a key role in the signaling pathway, resulting from activation of β_1 integrins, and that this enzyme may be required for up-regulation of cell-substrate and cell-cell adhesion.

2558

A Novel Autocrine Mechanism Activated By β1 Integrins That Supports Cell Adhesion Via IGF-II and Type 1 IGF Receptor

Loredana Moro[1], Mara Fornaro[1], Thomas L Mccarthy[2], Michael Centrella[2], Lucia R. Languino[1], [1]Pathology, Yale, [2]Surgery, Yale

Integrins and growth factor receptors are known to modulate signaling pathways either independently or synergistically. Using β1 integrin cell transfectants, we show that β1C, a β1 cytoplasmic variant, mediates cell adhesion to laminin-1, whereas the β1A cytoplasmic variant poorly supports cell adhesion to laminin-1. The increased cell adhesion to laminin-1 mediated by β1C is caused by upregulation of IGF (insulin-like growth factor) II at the mRNA and protein levels, and is significantly inhibited by antibodies to IGF-II and to the type 1 IGF receptor (IGF-1R). In β1C–cells, this increase in IGF-II causes PI3-kinase activation that, as evaluated by the use of dominant negative forms of PI3-kinase, is the only downstream signal that controls cell adhesion to laminin-1. In contrast, the β1A variant does not upregulate IGF-II levels and shows minimal binding to laminin-1. Finally, the IGF-1R shows the ability to associate with β1A, whereas it does not associate with β1C and is constitutively phosphorylated on tyrosine in β1C–expressing cells. In conclusion, we show that the β1 integrin extracellular binding domain weakly mediates cell adhesion to laminin-1 unless the following cascade of events is activated: upregulation of IGF-II levels and activation of PI3-kinase. Thus, we describe here a novel autocrine mechanism that supports cell adhesion via IGF-II and its binding partner, IGF–1R, and that is selectively regulated by the β1 integrin cytoplasmic domain in a PI3-kinase dependent manner.

¶Loredana Moro, present address: Center of Study on Mitochondria and Energy Metabolism, CNR, Bari, Italy

2559

Integrins Mediate Muscle Cell spreading Through PKC and MARCKS Signaling Pathway

Marie-Helene Disatnik[1], Stephane C Boutet[2], Thomas A Rando[3], [1]Department of Neurology/Neurological Sciences, Stanford University, 300 Pasteur Dr., Stanford,

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1750
Neurite Outgrowth Promotion by the Alternatively Spliced Region of Tenascin-C is Mediated by a Short Amino Acid Sequence and a Neuronal β1 Integrin
Sally Ann Meiners, Mohammed S.A. Nur-e-Kamal, Mary Lynn T. Mercado, Pharmacology, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854

Our work centers upon understanding how the extracellular matrix molecule tenascin-C regulates neuronal growth. The region of tenascin-C containing only alternately spliced fibronectin type-III repeat D (fnD) increases neurite outgrowth by itself and also as part of tenascin-C. We used overlapping peptides to localize the active site within fnD to an 8 amino acid sequence, VFDNFVLK. Of these, "FD" and "FV" are conserved in tenascin-C sequences derived from all the species available in the Gene Bank. A recombinant fnD protein and peptides with alterations in "FD" and/or "FV" did not facilitate process extension, supporting the hypothesis that the conserved residues are required for activity. We also found that a blocking antibody against β1 integrin completely abolished outgrowth promotion by VFDNFVLK. Hence a β1 integrin neuronal receptor apparently mediates promotion of neurite outgrowth by a non-RGD site in fnD (VFDNFVLK), much as a β1 integrin mediates cell attachment to a non-RGD site (AEIDGIEL) in the 3rd FN-III repeat of tenascin-C (Yokosaki et al., 1994). The crystal structure of fn3 has been reported (Leahy et al., 1992), and AEIDGIEL includes portions of the exposed B-C loop and adjacent C beta strand. Yokasaki et al. (1998) provide evidence that "D" in the exposed loop and "E" in the adjacent beta strand are both required for binding to the integrin α9β1. Alignment of the fn3 and fnD sequences reveals that EIDGIEL in fn3 corresponds to VFDNFVL in fnD. By extension, it is highly likely that "FD" found in VFDNFVLK is appropriately localized on an exposed loop in fnD for an interaction with neurons, whereas "FV" is likely localized on a semi-buried beta strand, where it may lend conformational stability rather than binding directly to neurons.

1751
Stromal Derived Factor-1 Activation of Extracellular Regulated Kinase in Jurkat T Cells is Regulated by Integrin-Mediated Adhesion
Tonya S. Laakko, Rudolph L. Juliano, Department of Pharmacology, University of North Carolina-Chapel Hill, CB#7365 Mary Ellen Jones Building, Chapel Hill, NC 27599

The importance of the chemokine, stromal derived factor-1 (SDF-1), in T lymphocyte function is rapidly becoming apparent. The SDF-1 receptor, CXCR4, which is found on T cells, has been implicated in homing, and as an HIV co-receptor and in metastasis. Considering the biological importance of the transiently adherent nature of T cells, little is known of the potential role that adhesion might play in regulating SDF-1 signal transduction to mitogen activated protein kinases (MAPK), such as extracellular regulated kinase (ERK), p38 and c-Jun n-terminal Kinase (JNK). Here we show, in Jurkat T cells, that beta-1 integrin-mediated adhesion promotes substantial SDF-1 activation of ERK, whereas cells in suspension demonstrated only minimal levels of SDF-1 mediated ERK activation. This response was transient, with maximal activation/phosphorylation apparent by Western analysis five minutes following treatment with the chemokine. Adhesion to immobilized fibronectin, vascular cell adhesion molecule-1 (VCAM-1) or the beta-1 integrin-activating antibody TS2/16 for one hour resulted in the efficient phosphorylation of ERK via SDF-1, thus indicating alpha-4 beta-1 and perhaps alpha-5 beta-1 integrins as regulators of activation. Interestingly, upon further investigation of the ERK MAPK cascade, we found that SDF-1 activation of the ERK kinase (MEK) demonstrated only limited dependence on integrin-mediated adhesion for activation. This suggests that in this system a rarely reported adhesion regulated control point between MEK and ERK is utilized. Preliminary analysis of both the p38 and JNK MAPK indicate constitutive activation that is not significantly enhanced by SDF-1 and/or adhesion. These data, therefore, support integrin-mediated adhesion in the efficient activation of ERK, but not MEK, in SDF-1 signaling in Jurkat T lymphocytes.

1752
αVβ3 Integrin Expression Upregulates cdc2 Which Modulates Cell Migration
Thomas D. Manes[1], Duo-Qi Zheng[1], Joseph Loftus[2], Amy S. Woodard[1], Lucia R. Languino[1], [1]Pathology, Yale University School of Medicine, 310 Cedar St., New Haven, CT 06520, [2]Mayo Clinic Scottsdale, AZ

The αVβ3 integrin has been shown to increase cell migration in vitro and in vivo. Ectopic expression of β3 in prostate cancer LNCaP cells, that constitutively express αV but not β3, increases LNCaP cell migration on integrin ligands, such as fibronectin and vitronectin. Gene expression analysis using cDNA arrays probed with first strand cDNA of mRNA isolated from either β3-LNCaP, mock-LNCaP or ICAM-LNCaP cell transfectants, showed increased cdc2 mRNA levels in cells expressing β3. Increased cdc2 protein levels and kinase activity were also observed in response to β3 expression both in two-dimensional and in Matrigel three-dimensional cultures. The effect was specific for αVβ3, since αVβ6 expression did not increase cdc2 levels. In addition, αVβ3-mediated upregulation of cdc2 was adhesion and ligand-binding independent, since expression of a ligand-binding mutant of β3 (D119A) equally increased cdc2 levels. Functionally, increased levels of cdc2 did correlate with increased migration on fibronectin of β3-LNCaP cells, as compared to β6-LNCaP cells. Several approaches were taken to demonstrate that cdc2 modulates LNCaP cell migration. Ectopic expression of dn-(dominant negative) cdc2 or a 2-hour treatment with either alsterpaullone or purvalanol A (cdc2 inhibitors) reduced LNCaP cell migration on fibronectin without affecting either adhesion or cell cycle progression. Finally, we show that ectopic expression of cdc2 increased cell migration. The effect of either dn-cdc2 or cdc2 was not cell-type specific, since similar results were observed in primary endothelial and HeLa cells. These results describe a new pathway that controls cell migration and that is modulated by both the αvβ3 integrin and its downstream target cdc2.

1753
Suppression of Tumour-Related Glycosylation, Cell Migration, and Adhesion by the 16K Membrane Subunit of V-ATPase
Mhairi A Skinner, Alan G Wildeman, Molecular Biology and Genetics, University of Guelph, Gordon St., Guelph, ON N1G 2W1 Canada

Integrins and other cell surface receptors are extensively glycosylated by enzymes resident in the endoplasmic reticulum (ER) and Golgi complex. Many cancer cells exhibit altered glycosylation patterns on surface receptors, and in specific cases hyperactivity of one of the enzymes responsible, N-acetylglucosaminyltransferase V (GlcNAc-TV) promotes a metastatic phenotype. β1 integrin is one of the molecules targeted by GlcNAc-TV, which adds N-acetylglucosamine (GlcNAc) to the oligosaccharide backbone via a β1,6 linkage. This widely expressed integrin heterodimerizes with most integrin subunits and is implicated in the invasive process of many tumor cells. We recently showed that the transmembrane domain of β1 integrin interacts with the 16K subunit of vacuolar H+ATPase (V-ATPase), the enzyme that acidifies the Golgi and exo- and endocytic compartments. 16K is a membrane-spanning protein that assembles into a hexamer forming the membrane proton channel of the enzyme. Since transmembrane domains play an important role in the trafficking of ER-routed proteins, as does pH, we examined whether 16K plays a role in β1 integrin processing. 16K was found to suppress β1,6 branching of β1 integrin in HEK293 cells. In addition, over-expression of 16K inhibited cell migration and cell adhesion. We also examined processing of the receptor for epidermal growth factor, and found that 16K similarly suppressed β1,6 oligosaccharide branching of this molecule. These data link cell surface tumor-related glycosylation to a component of the enzyme responsible for acidification of compartments of the exocytic pathway. In particular, 16K may provide opportunities for intervention in β1,6 branching and reduction of invasive cell growth.

1754
α4β1 Integrin Ectopically Expressed in CHO Cells Promotes Directional Protrusion of Lamellipodia α
Karen A Pinco, Wei He, Joy T Yang, Cell Biology, Johns Hopkins School of Medicine, 725 North Wolfe St., Baltimore, MD 21205

Although α4β1 integrin is known to be expressed in migratory cells in vivo (eg. neural crest cells, smooth muscle cells of newly-formed blood vessels, hematopoietic cell lineages) and has been shown to promote cell migration in vitro, it is not known how α4β1 promotes cell migration. The migration of adherent cells requires stabilization of lamellipodia in a polarized fashion leading to persistent migration in one direction. By scratch-wounding a cell monolayer to induce cell migration, we found that CHO cells ectopically expressing α4β1 integrin (CHO-α4 cells) protruded broad lamellipodia with persistent polarity toward the scratch-wound, whereas the CHO cells, expressing α5β1 but not α4β1 integrin, protruded membrane extensions randomly with little persistent polarity. The protrusion of broad lamellipodia by migrating CHO-α4 cells was inhibited by a functional blocking antibody against α4, indicating that α4β1 was required to maintain the broad lamellipodia. As the cytoplasmic tail of α4 has been shown to bind directly to paxillin, we generated a mutation (Y991A) in the cytoplasmic tail of α4 which has been shown to disrupt this interaction. In response to scratch-wounding, CHO cells ectopically expressing α4(Y991A) (CHO-α4(Y991A) cells) also protrude broad lamellipodia, but the cells migrated at a faster rate as compared to CHO-α4 cells. This result suggests that interactions between α4 and paxillin negatively contribute to the migration-promoting activity of α4β1. This work was supported by grants from the W.W. Smith Charitable Trust (#H9602) and the American Cancer Society (RPG-98-229-01-DDC).

1755
α4 Integrins and VCAM-1: Role in Sympathetic Innervation of the Heart
Kevin L. Wingerd, Nichol Goodman, Sergiu T. Leu, Matthew Smail, Steven X. Rohan, Dennis O. Clegg, Department of Molecular, Cellular and Developmental Biology, University of California

Sympathetic neurons innervate the heart early in postnatal development, an event which is crucial for proper modulation of blood pressure and cardiac function. However, the axon guidance cues that direct sympathetic neurons to the heart, and the neuronal receptors that recognize those cues, are poorly understood. Here we present evidence that interactions between the α4β1 integrin on sympathetic neurons and vascular cell adhesion molecule-1 (VCAM-1) in the heart plays a role in cardiac innervation.

Experimental Biology

2001

March 31-April 4, 2001
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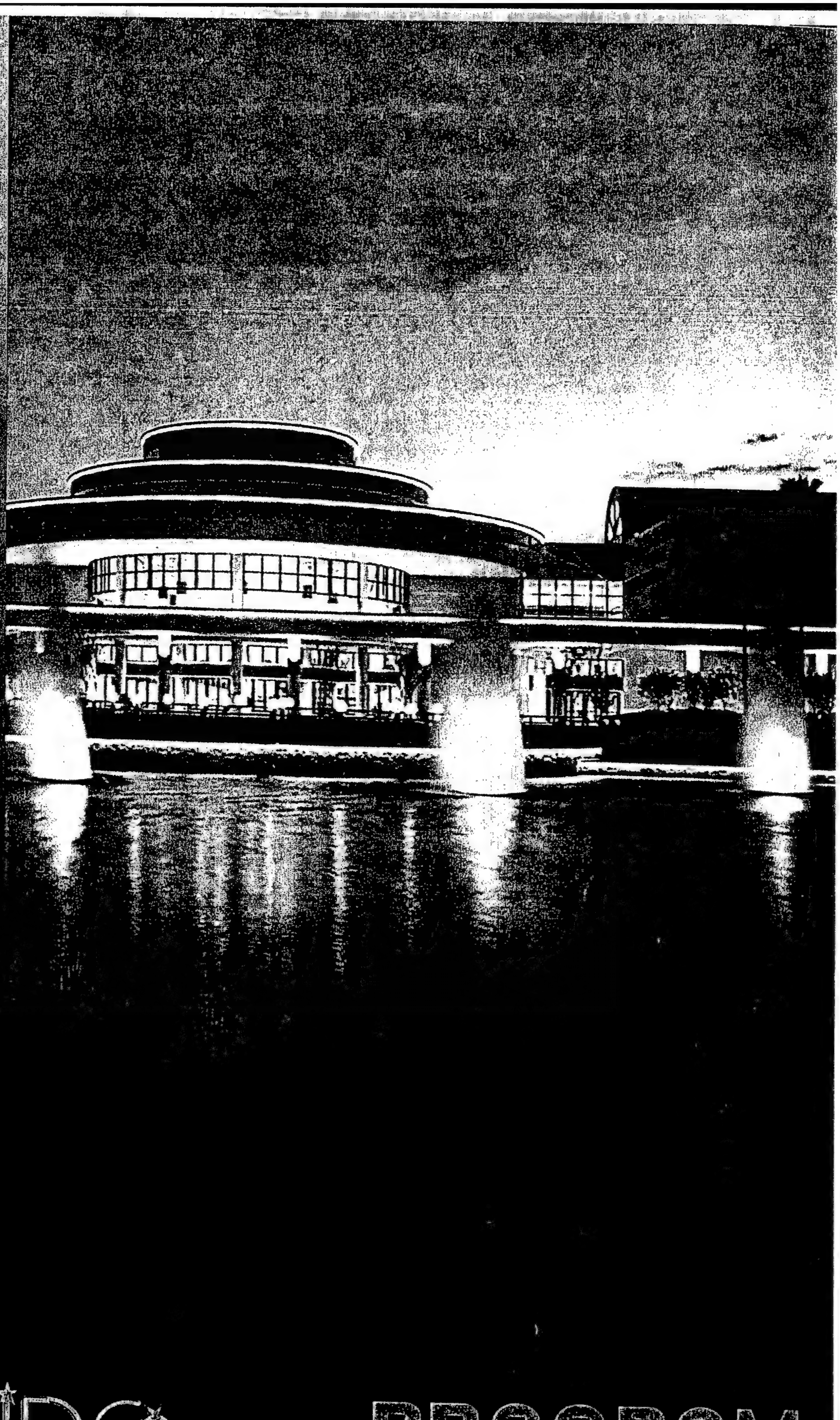

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PROGRAM

C-162 **212.10** Female BALB/c mice are more sensitive than males to a short-term repeated dose of fumonisin B_1. **N. Bhandari, Q. He and R.P. Sharma.** Univ. of Georgia.
C-163 **212.11** Metabolism of heterocyclic amines by mammalian enzymes: mutagen activation. **J.W. Gaubatz, W.S. Murph, Jr. and J.G. Dubuisson.** Univ. of South Alabama.
C-164 **212.12** Tanshinone I induces apoptosis via inhibiting NF-κB in activated hepatic stellate cells. **J-Y. Kim, J-X. Nan, K.M. Kim and D.H. Sohn.** Wonkwang Univ., Republic of Korea.
C-165 **212.13** Antiproliferative and pro-apoptotic effect of 5,6,7-trisubstituted flavones on a multidrug-resistant cell line. **M. Beviacqua, J.C. Calvo and A. Pomilio.** Inst.of Biol. and Exptl. Med. and PROPLAME-CONICET, Buenos Aires.
C-166 **212.14** Observations on the application of neutral red assay for cell viability. **R. Sridhar, K.V. Balan, Y. Zhou, R.A. Shankar and A.L. Goldson.** Howard Univ.
C-167 **212.15** Spectrophotometric assay of sodium, potassium-ATP-ase activity in the gills of small teleost fish by the ascorbic acid method. **E.G. Spokas, G.M. Cohen and P-S. Lai.** UMDNJ-Sch. of Osteo. Med. andTroy State Univ.
C-168 **212.16** Evaluation of EROD and AChE activity as biomarkers of exposure of Japanese medaka to 2,4-dinitrotoluene *in vivo* and *in vitro*. **P.O.O. Obih, I.N. Igbo and T.L. Huang.** Xavier Univ. of Louisiana.
C-169 **212.17** Genotoxic effects of industrial wastewater effluents in *Vicia faba*. **B.P. Patlolla, A.K. Patlolla and B.S. Sekhon.** Alcorn State Univ. and Jackson State Univ.
C-170 **212.18** Effects of tyrosine hydroxylase inhibition and gene mutations upon locomotor activity in *Drosophila*: a study in functional genomics. **R.G. Pendleton, A. Rasheed, T. Sardina and R. Hillman.** Temple Univ.

Pathology

213. PROTEASES IN VASCULAR BIOLOGY

Symposium

(Supported by an educational grant from Merck Research Laboratories and Bristol-Meyers Squibb Pharmaceutical Research Institute.)

SUN. 8:30 AM—ORANGE COUNTY CONVENTION CENTER, ROOM 206 B

Chaired: S.R. COUGHLIN

8:30 Platelet and endothelial cell function. **S.R. Coughlin.** Univ of California-San Francisco.
9:15 The endothelium as a regulator of the coagulation cascade. **C.T. Esmon.** Oklahoma Med Res Fndn.
10:00 Plasmin-mediated proteolysis in inflammation and repair. **J.L. Degen.** Children's Hosp Res Foundation.
10:45 Matrix metalloproteinases in angiogenesis. **Z. Werb.** Univ of California-San Francisco.

214. PROSTATE CANCER

Minisymposium

SUN. 8:30 AM—ORANGE COUNTY CONVENTION CENTER, ROOM 206 C

Chaired: S. COLLINS-PRESNELL

Cochaired: C. RINKER-SCHAEFFER

8:30 **214.1** Differential expression of hepatocyte growth factor and c-Met in prostate cancer amount Caucasian American versus African American patients. **S.C. Presnell, K. Borchert, C. Gregory, S. Maygarden, G. Smith and J. Mohler.** Univ. of North Carolina at Chapel Hill.
8:45 **214.2** Increased expression of hepatocyte growth factor, c-Met, and androgen receptor is associated with the transition from androgen-dependent to androgen-independent prostate cancer . **S.C. Presnell, K. Borchert, C. Gregory, S. Maygarden and J. Mohler.** Univ. of North Carolina at Chapel Hill.
9:00 **214.3** Stroma-epithelial interaction in prostate cancer as the basis for molecular co-targeting with adenoviral vectors. **L.W.K. Chung, A. Law, C-L. Hsieh, S. Matsubara and H. Rhee.** Univ. of Virginia Hlth. Sci. Ctr.
9:15 **214.4** Suppression of prostate tumor xenograft growth following localized treatment with TRAIL/Apo-2 expressing recombinant adenovirus. **T.S. Griffith, R.D. Anderson, B.L. Davidson, E.L. Broghammer, R.D. Williams and T.L. Ratliff.** Univ. of Iowa.
9:30 **214.5** Bcl-2 protects the human prostatic carcinoma cell line PC3 from TRAIL-mediated apoptosis. **M.B. Cohen, N.V. Guseva, A.F. Tagiev and O.W. Rokhlin.** Univ. of Iowa Col. of Med.
9:45 **214.6** AVβ3, an integrin up-regulated in prostate cancer, increases Cdc2 cyclin-dependent kinase levels. **T. Manes, D. Jain, D-Q. Zheng, A.S. Woodard, G. Tallini and L.R. Languino.** Yale Univ.
10:00 **214.7** Concordant proto-oncogene PML and HLA class I down-regulation in surgically removed prostate cancer lesions: an immunohistochemical study. **H. Zhang, J. Melamed, K. Cox, S. Ferrone, W.L. Frankel, R.R. Bahnson and P. Zheng.** Ohio State Univ., NYU Med. Ctr. and Roswell Park Cancer Inst.
10:15 **214.8** Immunotherapy of metastatic prostate cancer with low-dose total body irradiation. **M. Pollycove and L.E. Feinendegen.** US Nuclear Regul. Commission, Rockville MD and Brookhaven Natl. Lab.
10:30 **214.9** Thymic deletion of specific T cells reactive to SV40 large T antigen in TRAMP mice. **X.T. Zheng, J. Gao, T. Geiger, Y. Liu and P. Zheng.** Ohio State Univ. and St. Jude Children's Res. Hosp.
10:45 **214.10 (Moved to Session 220.)**

215. LIVER PATHOBIOLOGY: GENE THERAPY OF LIVER DISEASE

Workshop

SUN. 8:00 AM—ORANGE COUNTY CONVENTION CENTER, ROOM 206 A

Chaired: T.R. FLOTTE

Cochaired: B.E. PETERSEN

8:00 Liver stem cells as potential platforms for gene therapy. **B.R. Petersen.** Univ of Florida.
9:00 Alpha 1-antitrypsin deficiency: a model of gene therapy for both recessive and dominant disorders. **T.R. Flotte.** Univ of Florida Col of Med.

214.5

Bcl-2 Protects the Human Prostatic Carcinoma Cell Line PC3 from TRAIL-mediated Apoptosis

Michael B. Cohen, Natalie V. Guseva, Agshin F. Tagiev, Oskar W. Rokhlin: The University of Iowa College of Medicine, Department of Pathology, 200 Hawkins Drive, Iowa City, IA 52242

Bcl-2 protects the human prostatic carcinoma cell line PC3 from TRAIL-mediated apoptosis

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor family and has recently been shown to induce apoptosis in cancer cells in vivo without observable toxicity. However, the signaling pathways triggered by TRAIL stimulation have not been investigated in human prostate cancer. Here, we investigated TRAIL-induced apoptosis in the human prostatic carcinoma cell line PC3 and in a PC3 subline overexpressing Bcl-2. Cell death was estimated in both cell lines by calcein assay and crystal violet staining. Apoptosis was estimated by DNA laddering, DEVDase activity, proteolysis of PARP and DFF-45, and western blot analysis of caspases-2,-3,-7, -8, and -9 both in the cytosol and in isolated mitochondria. We have also investigated mitochondrial transmembrane potential (MTP) and cytochrome c release from mitochondria. PC3 was found to be highly sensitive to TRAIL-induced apoptosis whereas PC3-Bcl-2 revealed a high level of resistance. TRAIL was found to activate initiator caspases-2, -8, and -9 as well as executioner caspases-3 and -7. In addition, TRAIL-mediated apoptosis involves dissipation of MTP and caspase-dependent cytochrome c release. Comparative investigation of PC3 and PC3-Bcl-2 revealed that Bcl-2 overexpression attenuated activation of all caspases in the cytosol. In contrast to these quantitative differences, activation of mitochondrial localized caspases-2 and -9 were completely prevented by Bcl-2 overexpression. Bcl-2 also completely abrogated TRAIL-induced cytochrome c release and dissipation of MTP in PC3-Bcl-2. Finally, we did not find any differences between PC3 and PC3-Bcl-2 in Bid processing under TRAIL treatment. Taken together, these findings suggest that TRAIL-induced apoptosis in PC3 depends on mitochondrial integrity rather than on caspase activation.

214.6

$\alpha V\beta 3$, AN INTEGRIN UPREGULATED IN PROSTATE CANCER, INCREASES CDC2 CYCLIN DEPENDENT KINASE LEVELS

Thomas Manes, Dhanpat Jain, Duo-Qi Zheng, Amy S Woodard, Giovanni Tallini, Lucia R. Languino: Yale

The $\alpha V\beta 3$ integrin has been shown to modulate several cellular functions, including migration, anchorage-dependent and -independent growth, as well as survival. Its expression is up-regulated in prostate cancer and in metastatic prostate carcinoma cells, whereas normal prostate tissue appears largely negative. Ectopic expression of the $\beta 3$ integrin in prostate cancer LNCaP cells that constitutively express αV, but not $\beta 3$, generates a functional $\alpha V\ \beta 3$ integrin which increases these cells' proliferation. To examine the effect of $\alpha V\beta 3$ integrin on gene expression in LNCaP cells, cDNA arrays probed using first strand cDNA of mRNA isolated from either $\beta 3$ ($\beta 3$-LNCaP), vector (mock-LNCaP) or ICAM-1 (ICAM-LNCaP) cell transfectants were compared. Upregulation of cyclin-dependent kinase cdc2 mRNA levels was observed in cells expressing $\beta 3$ when compared to cells expressing ICAM-1 or to cells that were mock-transfected. Increased cdc2 protein levels and kinase activity were also observed in response to $\beta 3$ expression in $\beta 3$-LNCaP cells. Cdc2 protein and kinase activity levels were higher in $\beta 3$-LNCaP cells compared to ICAM-LNCaP or mock-LNCaP cells both in two-dimensional and in Matrigel three-dimensional cultures. The upregulation of cdc2 was due to $\alpha V\beta 3$ expression but was independent of $\alpha V\beta 3$ integrin engagement by its ligand vitronectin. Furthermore, $\alpha V\beta 3$'s effect on cdc2 protein levels was not synergistically increased by serum stimulation. In addition to the previously known role of $\alpha V\beta 3$ in mediating migration of LNCaP cells, these data highlight a new role for the $\alpha V\beta 3$ integrin in an adhesion-independent control of prostate cancer cell cycle molecule expression and activities.

214.7

Concordant Proto-oncogene PML and HLA Class I Down-regulation in Surgically Removed Prostate Cancer Lesions: An Immunohistochemical Study

Huiming Zhang[1], Jonathan Melamed[2], Karen Cox[1], Soldano Ferrone[3], Wendy L Frankel[1], Robert R Bahnson[1], Pan Zheng[1]: [1]The Ohio State University, 158 HH, 1645 Neil Avenue, Columbus, OH 43210, [2]New York University Medical Center, 550 First Avenue, New York, NY 10016, [3]Roswell Park Cancer Institute, Elm and Carlton Street, Buffalo, NY 14263

Antigen peptides presented by the class I major histocompatibility complex (MHC) molecules are primary targets on tumor cells for immune recognition by host cytotoxic T lymphocytes (CTL). As a result MHC class I down-regulation which is frequently found in malignant tumors has a negative impact on their recognition by T cells. Normal cell surface MHC class I expression requires coordinated expression of multiple genes encoding, respectively, proteosome components LMP2/7, peptide transporters TAP1/2, β_2 microglobulin(β_2M) and MHC class I heavy chain. We have previously reported that proto-oncogene product PML induces expression of TAP1, TAP2, LMP2 and LMP7 in an MHC class I negative, recurrent tumor, leading to the re-expression of cell surface MHC class I in tumors and to rejection of tumors (Nature, 396:373-376). In this study, we examined the expression of proto-oncogene product PML expression and of HLA class I antigens in 37 surgically removed prostate carcinoma lesions. Immunohistochemical staining of formalin fixed paraffin embedded sections with anti-HLA class I heavy chain monoclonal antibody (mAb) HC10 detected their down-regulation in 27 lesions (73%) with different extent (50-90% of carcinoma cells were not stained by mAb HC10). Furthermore immunohistochemical staining with anti-PML mAb PG-M3 showed that 23 of the 27 lesions (85%) with HLA class I antigen downregulation had also down-regulation of PML nuclear expression (17 cases with complete lack of reactivity to PG-M3 and 6 cases with weak reactivity to PG-M3). Morphologically, the Gleason grade 3C carcinoma that consists of well circumscribed cribriform tumor mass is the most common type that exhibits simultaneous complete loss of the HLA class I and PML expression. In summary, our results suggest that PML down-regulation is strongly associated with HLA class I down-regulation in prostate cancer.

214.8

Immunotherapy of Metastatic Prostate Cancer with Low-Dose Total Body Irradiation (LDR)

Myron Pollycove[1], Ludwig E. Feinendegen[2]: [1]U.S. Nuclear Regulatory Commission, 11555 Rockville Pike 016-E15, Rockville, MD 20852, [2]Brookhaven National Laboratory

Observations of mice, rats, and human clinical trials demonstrate the efficacy of low-dose x-ray or gamma ray radiation immunotherapy of cancer using single doses of 15 or 20 rad or fractionated doses of 4-15 rad in 1-2 month courses of 48-150 rad. The immune system is an essential component of effective antimutagenic control of the enormous burden of relentless metabolic DNA alterations produced by reactive oxygen species (ROS) leaked from mitochondria. The human antimutagenic biosystem includes antioxidant prevention, enzymatic repair of DNA alterations and removal of persistent DNA alterations by apoptosis and the immune system that together reduce DNA damage from 1,000,000 DNA alterations/cell/d to ~1 "mutation"/cell/d.

Recent research has led to recognition of the importance of immune surveillance in controlling cancer as well as infectious disease. High doses of radiation suppress the immune system but low doses stimulate the production of CD8+ cytotoxic lymphocytes with prevention and regression of tumor metastases. Whole body irradiation of mice with a single dose of 15r increased the effectiveness of a cancer vaccine more than a thousandfold. Published results demonstrating effectiveness superior to chemotherapy justify support of well-designed clinical trials of LDR therapy in patients with metastatic prostate cancer or for prevention of metastases following initial surgical or radiation therapy of prostate cancer. Understanding of mechanistic details of the stimulatory response of the immune system to body irradiation is needed to develop optimal clinical protocols for prostate cancer therapy.

214.9

Thymic Deletion of Specific T Cells Reactive to SV40 Large T Antigen in TRAMP Mice

Xincheng Ted Zheng[1], Jianxin Gao[1], Terrence Geiger[2], Yang Liu[1], Pan Zheng[1]: [1]The Ohio State University, 158 Hamilton Hall, 1645 Neil A, Columbus, OH 43210, [2]St. Jude Children's Research Hospital, 332 North Lauderdale, Memphis, TN 38105

The transgenic adenocarcinoma of mouse prostate (TRAMP) model is transgenic for the SV40 large T antigen (Tag) under the control of the rat probasin regulatory elements. The TRAMP mice develop tumors spontaneously and orthotopically with a disease progression that closely resembles the progression of human prostate cancer. Previous studies showed that T lymphocytes from TRAMP mice are immune tolerant to SV40 Tag, while the mechanism of the tolerance is not clear. In this study, we immunized the TRAMP mice with an immunodominant SV40 Tag epitope IV (peptide 404-411) which is presented by the class I major histocompatibility complex molecule H-2K^b, and analyzed the antigen specific T cell response by ELISPOT. We could not detect any antigen specific T cell response to Tag epitope IV in TRAMP mice, which was in consistent with the previous report. To examine whether the immune tolerance is due to thymic deletion, we crossed the TRAMP mice with TG-B mice transgenic for a rearranged T-cell receptor that recognizes Tag (peptide 559-576) presented by the class I major histocompatibility complex molecule H-2K^k. Double transgenic TRAMP/TCR mice had thymic deletion of SV40 Tag reactive T cells when examined at 25 days after birth. The thymus size is reduced from 6.5×10^7 thymocytes in TCR transgenic mouse to 5×10^6 thymocytes in double transgenic mouse. The mature CD8+V$_\beta$8+ T cells from spleen are reduced from 5×10^6 cells in TCR transgenic mouse to 1×10^5 cells in double transgenic mouse that possibly represent the endogenously rearranged V$_\beta$8+ T cells. The thymic deletion of SV40 Tag specific T cells is identified in both male and female double transgenic TRAMP/TCR mice. We subsequently detected the message for the SV40 Tag in the thymus of the double transgenic TRAMP/TCR mice and TRAMP mice through RT-PCR and Southern blot. Our study showed that thymic deletion of T cells specific for SV40 Tag is the major mechanism for T cell tolerance in TRAMP mice.

214.10

BD.1, A Ductal Protein Associated With Preneoplasia in Rat Prostate Epithelial Cells

Tara Lee Frenkl[1], Angela C McBride[1], Marie Carreiro[1], Li Yang[2], Douglas C. Hixson[1]: [1]Rhode Island Hospital, 593 Eddy Street, Providence, Rhode Island 02903, [2]Emory University, Atlanta, GA

BD.1 is a monoclonal antibody defined protein that is expressed by normal rat bile duct epithelial cells but not oval cells. Previous in vitro studies on a continuous line of low passage bile duct epithelial cells (BDE1.1) revealed that BD.1 expression greatly increased when cycling cells were blocked with methotrexate at the G1/S boundary but rapidly declined following release and subsequent arrest by nocodazole in G2/M. When the same experiments were performed on high passage BDE1.1 and a continuous line of oval cells (CDE6), basal levels of expression were low or undetectable and no induction occurred following treatment with methotrexate. These data suggested that loss of BD.1 induction could be a marker for early neoplasia. Since prostate epithelial cells (PEC) express bile duct markers including BD.1, it was of interest to determine whether low passage PEC and SV40 LT immortalized PEC SV40LT-PEC) would differ in their expression of BD.1. G1/S arrest of PEC with methotrexate or mimosine (verified by the pattern of PCNA nuclear staining) revealed a significant increase in expression (2.5-4 fold) of BD.1 expression levels as determined by indirect immunofluorescence or ELISA. In contrast, basal levels of BD.1 were significantly lower on SV40LT-PEC and cycle arrest in G1/S did not induce expression. These observations suggest that the loss of BD.1 inducibility could signal an early event in the neoplastic process. This work was supported by NCI Grant CA42715.

Pathology

321. PFIZER OUTSTANDING INVESTIGATOR AWARD LECTURE

SUN. 5:30 PM—ORANGE COUNTY CONVENTION CENTER, ROOM 206 A

Award Recipient: **R.W. Doms**, Univ. of Pennsylvania.

Title: Closing the Door on HIV Entry

322. CHUGAI AWARD FOR MENTORING AND CHUGAI SYMPOSIUM FOR YOUNG INVESTIGATORS

SUN. 2:00 PM—ORANGE COUNTY CONVENTION CENTER, ROOM 205 A

Chaired: J.A. MADRI

Award Recipient: **J.A. Madri**, Yale Univ.

2:00 PECAM-1: a multidomain/multifunctional protein with diverse signaling and scaffolding properties - implications for angiogenesis and inflammation. **J. Madri.** Yale Univ. Sch. of Med.

2:45 Differential tyrosine dephosphorylation of platelet endothelial cell adhesion molecule directly modulates the phosphatase activity of associated SHP2. **D.A. Gratzinger, M. Barreuther, A. Tucker and J.A. Madri.** Yale Univ. Sch. of Med. and Albertus Magnus Col., Ct. **(730.12)**

3:00 Development of radioligands for *in vivo* imaging of brain amyloid. **D.M. Skovronsky, M-P. Kung, B. Zhang, H. Kung, J.Q. Trojanowski and V.M-Y. Lee.** Univ. of Pennsylvania. **(726.4)**

3:15 Live-time imaging of vascular endothelial-cadherin during leukocyte transmigration across endothelium. **S.K. Shaw, P.S. Bamba, B.N. Perkins and F.W. Luscinskas.** Brigham and Women's Hosp. **(924.4)**

3:30 AVβ3, an integrin up-regulated in prostate cancer, increases Cdc2 cyclin-dependent kinase levels. **T. Manes, D. Jain, D-Q. Zheng, A.S. Woodard, G. Tallini and L.R. Languino.** Yale Univ. **(214.6)**

3:45 A recombinant human Fab inhibits helicase activity of HCV NS3 and negative strand RNA synthesis. **N. Khalap, R. Burioni, M. Clementi and S. Dash.** Tulane Univ. Med. Sch., Univ. of Ancona and Univ. of Trieste, Italy. **(473.4)**

4:00 CD99 is used for transendothelial migration of monocytes. **A.R. Schenkel, R.M. Liebman, X. Chen and W.A. Muller.** Weill Med. Col. of Cornell Univ. **(924.3)**

4:15 Gene expression profiling of keratinocyte differentiation by fluorescent differential display PCR. **D. Ranamukhaarachchi, M.S. Rajeevan, D.R. Lee, D.K. Williams, S.D. Vernon and E.R. Unger.** Ctr. for Dis. Control. **(927.7)**

4:30 The cyclooxygenase-2 (COX-2) inhibitor NS-398 selectively suppresses *in vitro* cell growth and induces apoptosis in C611B rat cholangiocarcinoma cells over-expressing COX-2. **Z. Zhang and A.E. Sirica.** Virginia Commonwealth Univ. **(925.9)**

4:45 **322.8** Effect of plasminogen activator inhibitor (PAI)-1 and PAI-3 on MDA-MB-435 breast tumor cell proliferation. **B.R. Whitley, D. Palmieri and F.C. Church.** Univ. of North Carolina at Chapel Hill. **(220.16)**

323. LEUKOCYTE-ENDOTHELIAL CELL INTERACTIONS

Poster Discussion

SUN. 2:00 PM—ORANGE COUNTY CONVENTION CENTER, ROOM 206 C

Chaired: M. CYBULSKY

Cochaired: S. NOURSHARGH

2:00 Welcome and poster viewing.

3:00 **323.1** Neutrophil elastase: surface expression and functional role in transmigration *in vivo*. **K.E. Noble, R.D. Thompson, K.Y. Larbi, A. Belaaouaj, S. Shapiro and S. Nourshargh.** Imperial Col. Sch. Of Med., London and Washington Univ. Sch. of Med.

3:10 **323.2** Murine neutrophil transendothelial migration under flow *in vitro* does not require elastase or MMP-9. **J.R. Allport, Y-C. Lim, J.M. Shipley, R.M. Senior, S.D. Shapiro, D. Vestweber, N. Matsuyoshi and F.W. Luscinskas.** Massachusetts Gen. Hosp., Charlestown, Brigham and Women's Hosp., Washington Univ. Sch. of Med., Univ. of Munster and Kyoto Univ.

3:20 **323.3** The role of α6 integrin in leukocyte migration *in vivo*. **J.P. Dangerfield, K.Y. Larbi, K.E. Noble, R.D. Thompson, A. Dewar and S. Nourshargh.** Imperial Col. Sch. of Med. and Royal Brompton Hosp., London.

3:30 **323.4** Transmigration-independent effects of neutrophils on epithelial barrier function. **H.A. Edens, A. Nusrat and C.A. Parkos.** Emory Univ.

3:40 **323.5** Demonstration of CAP37, a monocyte chemoattractant in endothelial cells. **T.D. Lee, P. Kumar, S. Chary-Reddy, P. Grammas and H.A. Pereira.** Univ. of Oklahoma Hlth. Sci. Ctr.

3:50 **323.6** Lack of protective effect of estrogen in eNOS-deficient mice. **A.J. Prorock, A. Hafezi-Moghadam, V.E. Laubach, J. Liao and K.F. Ley.** Univ. of Virginia and Harvard Univ.

4:00 **323.7** Thrombin induced leukocyte recruitment via NFκB and p38 MAP kinase pathways. **J. Kaur and P. Kubes** Univ. of Calgary, Canada.

4:10 **323.8** Down-regulation of human umbilical vein endothelial cell (HUVEC) NFκB by PMN results in a suppressed proadhesive phenotype in HUVEC. **G. Cepinskas, V. Savickiene, C.W. Lush and P. Kvietys.** Lawson Hlth. Res. Inst.

4:20 **323.9** P-selectin glycoprotein ligand-1 microsphere adhesion *in vivo*. **D.J. Goetz, E.E. Burch, M.F. Kiani and V.R. Shinde Patil.** Ohio Univ. and Univ. of Tennessee Hlth. Sci. Ctr. Sch. of Biomed. Engin.

4:30 **323.10** Leukocyte rolling velocity is determined by wall shear rate, not wall shear stress. **M.L. Smith, M.J. Smith, M.B. Lawrence and K.F. Ley.** Univ. of Virginia Hlth. Sci. Ctr.

4:40 **323.11** Ontogeny of Mac-1-dependent leukocyte localization in a rabbit model. **M.M. Mariscalco, J. Mei and C.W. Smith.** Baylor Col. of Med.

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Special Presentation

8:50 **Integrin, Growth Factor, and Tumor Suppressor Collaborations that Regulate Cell Signaling and the Cytoskeleton**

Kenneth M. Yamada, M.D., Ph.D., Branch Chief, **National Institute of Dental Research, National Institutes of Health**

Integrins can regulate the cytoskeleton, many major signal transduction pathways, and cell proliferation. They induce multi-molecular transmembrane complexes of cytoskeletal and signaling molecules, and rapidly induce numerous novel genes. Integrins signal via multiple pathways including MAP kinases, and they synergize with growth factors. Recombinant chimeras are helping to dissect these processes. The newly described tumor suppressor PTEN/MMAC1 regulates integrin-mediated cell spreading, migration, and adhesions. PTEN may prevent multiple human cancers by down regulating the key signaling molecules FAK, Shc, and MAP kinases. These collaborations between integrin/growth factor signaling pathways and a tumor suppressor provide many intriguing targets for potential therapeutic intervention.

9:30 **Signaling by Integrins in Prostate Cancer**

Lucia R. Languino, Ph.D., Assistant Professor, Pathology Department, **Yale University**

The interactions between cancer cells and the extracellular matrix are mediated by integrins, that have emerged as key regulators of cell proliferation, migration and intracellular signaling. Using genetically engineered and primary prostate cancer cells, we have found that the b_1 and b_3 integrins modulate growth and motility of prostate cancer cells. We will discuss the downstream intracellular signaling events modulated by these integrins and the pathophysiological relevance of their aberrant expression in prostate cancer. Our findings point to b1 and b_3 integrins as potential target molecules for novel therapeutic approaches in prostate cancer.

10:00 **Integrin Linked Kinase (ILK)**

Shoukat Dedhar, Ph.D., Professor, Biochemistry, **University of British Columbia and BC Cancer Research Center, Jack Bell Research Center**

The Integrin Linked Kinase (ILK) is an ankyrin repeat, and phosphoinositide lipid-binding motif, containing serine/threonine protein kinase. ILK can interact with the cytoplasmic domains of beta 1,2 and 3 integrin subunits. ILK activity is rapidly stimulated upon adhesion of cells to fibronectin in a Pi(3)K- dependent manner. The phosphoinositide, Pi(3,4,5)P3 can stimulate the kinase activity of ILK in vitro. Adhesion of epithelial cells to fibronectin results in the Pi3K dependent activation of the anti-apoptotic kinase, PKB/AKT, and in inhibition of the pro-apoptotic kinase, GSK-3. Transient or stable overexpression of ILK results in the constitutive activation of PKB/AKT, and inhibition of GSK-3. ILK can directly phosphorylate PKB/AKT on ser-473, which is a requirement for PKB activation. The inhibition of GSK-3 results in nuclear translocation of beta-catenin and the activation of the transcription factor, lef-1/beta-catenin. ILK also stimulates the transcription factor, AP-1. Co-transfection of wild type, active, GSK-3 reverses the ILK induced AP-1 activity, as does co-transfection of kinase-dead ILK. GSK-3 normally phosphorylates c-Jun at a site that results in the inhibition of Fos-Jun interaction and thus inhibition of AP-1. The inhibition of GSK-3 by ILK prevents this phosphorylation, resulting in the activation of AP-1. Thus ILK is an integrin proximal effector involved in the regulation of cell survival and cell growth pathways.

10:30 *Poster/Exhibit Viewing and Refreshment Break*

11:00 **Integrin Signaling: Lessons from the Immune System**

Yoji Shimizu, Ph.D., Department of Laboratory Medicine and Pathology Center for Immunology, **University of Minnesota Medical School**

In the immune system, signs of distress in the host result in lymphocyte activation. One of the earliest functional responses of an activated lymphocyte is a rapid, but transient, increase in integrin-mediated adhesion. Many receptors that activate integrins on T cells do so via activation of the lipid kinase phosphoinositide 3-OH kinase (PI 3-K). This paradigm of integrin activation also applies to growth factor receptor regulation of integrin function on tumor cells. Integrin engagement also leads to the generation of intracellular signals, and the relevance of some of these signals to the function of immune cells will be presented.

11:30 **A Requirement for Caveolin-1 and Associated Tyrosine Kinase Fyn in Integrin Signaling and Anchorage-Dependent Cell Growth**

Filippo G. Giancotti, M.D., Ph.D., Associate Member, Cellular Biochemistry and Biophysics Program, **Memorial Sloan-Kettering Cancer Center**

Previous studies have suggested that the adapter protein Shc plays a crucial role in integrin signaling. We provide evidence that caveolin-1, a protein previously implicated in the biogenesis of caveolae, functions as a membrane adapter to link integrins to the tyrosine kinase Fyn, thereby mediating recruitment of Sch. Immunoflourescent analyses indicate that a fraction of caveolin-1 coalignes with integrins at extracellular matrix contact sites. Co-immunoprecipitation and mutagenesis experiments provide evidence that caveolin-1 physically and functionally couples the transmembrane segment of integrin a subunit to Fyn. Mutational studies indicate that, upon integrin-mediated activation, Fyn binds via its SH3 domain to Shc. Shc is subsequently phosphorylated at tyrosine 317 and combines with Grb2. By introducing caveolin-1 in caveolin-1-negative epithelial cells and Fyn and mutants thereof in Fyn -/- fibroblasts, we demonstrate that this sequence of events is necessary to couple integrins to the Ras-ERK signaling pathway and promote progression through the G1 phase of the cell cycle. These findings reveal an unexpected function of caveolin-1 and illustrate a novelmechanism by which tyrosine kinase-mediated signaling controls anchorage-dependent cell growth.

12:00 *Lunch on your own*

1:25 **Chairperson's Remarks**

Lucia R. Languino, Ph.D., Assistant Professor, Pathology Department, **Yale University**

ADHESION IN MIGRATION

1:30 **Highly Stoichiometric, Stable, and Specific Association of Integrin Ó3β1 with CD151 Provides a Major Link to Phosphatidylinositol 4-Kinase, Regulate Cell Migration**

Martin E. Hemler, Ph.D., Professor, **Dana-Farber Cancer Institute**

The Ó3β1 integrin associates with transmembrane-4 superfamily (TM4SF) protein CD151. Association is highly stoichiometric (including nearly 90% of Ó3β1), is very stable (i.e. maintained in stringent detergents), and is highly specific (i.e. observed in absence of other cell surface proteins). Most of the abundant PtdIns 4-kinase activity associated with Ó3β1 is removed upon immunodepletion of CD151. Specificity for CD151 and PtdIns 4-kinase association resides in a Ó3β1 extracellular domain, thus establishing a novel paradigm for specific recruitment of an intracellular signaling molecule. Finally, antibodies to either CD151 or Ó3β1 caused a ~88-92% reduction in fMLP-induced, β-glucan stimulated neutrophil motility, thus suggesting a role of these complexes in cell migration.

2:00 **Some Steps in Cell Migration**

Alan Rick Horwitz, Ph.D., Professor, Department of Cell and Structural Biology, **University of Illinois**

Cell migration plays a central role in several normal and pathological processes including inflammation, wound repair, and tumor invasion and metastasis. It consists of a cycle of events that begins with an initial protrusion at the cell front, which is followed by stabilization of adhesion, movement of the cell cortex, and release of adhesions at the cell rear. Calcium transients, phosphatases, tension and calpain mediate release at the cell rear. Integrin signaling also regulates migration. Mutations that inhibit integrin signaling also inhibit migration. Effects of integrin signaling include FAK, Cas(crk), and MAP kinase. We are identifying the processes that these effectors

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